THE PHYSIOLOGICAL AND ANTIGENIC PROPERTIES OF BLOOD PLATELETS

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NP = Native plasma
RCP = Recalcified citrate plasma

Introduction

The experimental work to be described in this thesis was performed while investigating the role of immune mechanisms in the pathogenesis of Idiopathic Thrombocytopenic Purpura. The problem was first examined using conventional immunological techniques, as, since Evans & Duane (1950) drew attention to the similarity in behaviour between acquired haemolytic anaemia of immune origin and idiopathic thrombocytopenic purpura, many workers have claimed to have demonstrated specific platelet antibodies or agglutinins in blood derived from cases of idiopathic thrombocytopenia.

The methods used by individual investigators have varied but, while apparently successful and reliable in the hands of their originators, many have not produced reliable results in other centres. The majority have based their methods on conventional red blood cell agglutination techniques, and, having substituted platelets for red cells, have observed the presence or absence of platelet agglutination in varying systems. But unlike red blood cells, platelets clump as part of their normal physiological role in the process of blood coagulation. When whole blood is removed from the body and allowed to clot in vitro, the platelets clump or agglutinate and eventually undergo lysis as fibrin is formed.

Therefore, the initial step in this investigation was to re-examine this latter process in the light of modern knowledge

in order to determine, if not the exact nature, the properties of the factor or factors in blood coagulation which controlled platelet agglutination.

If platelet agglutination could be induced specifically by immune agglutinins in cases of thrombocytopenic purpura, presumably the mechanism must be different from that which occurs during clotting and thus at least two types of agglutination must exist.

As the techniques used to determine the presence or absence of platelet agglutination in vitro necessitated separating platelets from the other cellular elements of the blood and, in some instances, from their own plasma, it was also essential to decide what factors might alter or clump the platelets non-specifically during these procedures.

By applying this information it was possible to decide on a theoretically optimum technique for demonstrating specific platelet agglutinating factors in the blood derived from cases with unexplained thrombocytopenia. This established, it was essential to discover whether such specific platelet agglutinating factors might exist in normal blood; then it was possible to proceed to the examination of blood derived from cases of thrombocytopenia, with or without purpura, to confirm, if possible, the existence of platelet agglutinating factors in such cases, their relationship to the type and duration of the disease, and the effect of therapy on those cases with demonstrable anti-platelet factors in their blood.

The work incorporated in this thesis has been carried out since January 1955 during the tenure of the post of Graduate Assistant in the Department of Haematology, Radcliffe Infirmary, Oxford (Dr. R.G. Macfarlane, F.R.S.).

All the experimental work has been performed by myself without any technical assistance. Certain reagents have been provided and certain blood groups determined by other workers in the department and due acknowledgement is made for this assistance. The diagnoses of the specific defects of blood coagulation factors in the blood of the many patients used in the following experiments were made by Dr. Rosemary Biggs.

The experimental work of this thesis is unpublished with the exception of the following:

(i) The experiments relating to Complement and viscous metamorphosis described in Chapter IV have been incorporated in a letter to the Editor of NATURE on 'The Relationship of Complement to Blood Coagulation.' Sharp, A.A. (1957). Nature, Lond., 179, 632.

(ii) The experiments describing the effect of animal antihaemophilic globulin on human and other species' platelets have been described in a paper entitled 'The Toxicity and Fate of Injected Animal Antihaemophilic Globulin.' Sharp, A.A. and Bidwell, E. (1957). Lancet, ii, 359.

(iii) The entire experimental data in Chapter III and certain experiments from Chapter IV have been incorporated in a paper entitled 'Platelet Viscous Metamorphosis - A study of the relationship to fibrin formation.' Sharp, A.A. (1958). Brit. J. Haemat. In press.
(This latter paper was accepted by Professor J.V. Dacie in June 1957 and will be published in January 1958).

The photographs used to illustrate certain aspects of the experimental work of this thesis were printed from negatives prepared by myself.

PART I

cellular elements of the blood. This discovery was soon confirmed by Hayem (1878, 1882, 1883) and Bizzozero (1882) and it revolutionised the experimental approach to blood coagulation. Here at last was some factor, necessary for blood coagulation, that could be observed by the naked eye or with the aid of a microscope. Both Hayem (1878) and Bizzozero (1882) observed the striking property of platelets to clump and disintegrate in association with the clotting of the blood, and they inferred that the platelets were an essential part of the mechanism of coagulation. These opinions were questioned by Eberth & Schimmelbusch (1888) who pointed out that, if the platelets and platelet clumping were an integral part of fibrin formation, intravascular platelet clumping should provoke fibrin formation in the adjacent blood; this sequence they did not observe.

Almost simultaneously with the recognition of blood platelets, and their suggested role in blood coagulation, it was realised that cellular and tissue debris played an important role in blood clotting in wounds and it was shown that these products, when introduced into the blood stream, would produce intravascular clotting (Wooldridge 1883 a & b, 1889).

Yet another important milestone in the history of blood clotting followed with the discovery of the importance of the role of ionised calcium in blood coagulation. This discovery led to the 'classical theory' of blood coagulation

coagulation proposed by Morawitz (1905). The latter believed four factors must be present before fibrin formation could occur and his theory may be represented diagramatically by the following:

'Thromboplastin' + Calcium + Prothrombin

1 ↓

Thrombin

Thrombin + Fibrinogen

2 ↓

Fibrin

'Thromboplastin', Morawitz considered, was derived from either the blood platelets or the tissues. This 'classical' theory started an era in blood coagulation research which lasted for more than three decades. In this period evidence for and against the participation of platelets in blood coagulation was advanced and new theories of the mechanism of blood coagulation were propounded but not accepted universally.

Wooldridge (1889) considered that platelets were not independent elements of the blood but precipitated globulins, and that they had no function in the mechanism of blood coagulation. The work of Kemp (1884) and Burker (1904), however, established both the independent nature of blood platelets and their role in blood coagulation. When

Wright (1910) suggested that platelets originated from the megakaryocytes of the bone marrow, their presence as individual elements of the blood was accepted by the majority of workers in this field.

Alternatives to the 'Classical Theory' were proposed.

Theory of Bordet

Bordet & Delange (1912), postulating a 4-factor theory of blood coagulation, considered that there was an inert precursor "proserozyme" in the blood, which was activated to "serozyme" in the presence of calcium; this process being initiated by 'contact' with a foreign surface. Serozyme then, they thought, reacted with cytozyme to form thrombin which in turn converted fibrinogen to fibrin. Cytozyme or thromboplastin, they considered, originated in the tissue or the platelets.

Other theories at variance with those of Morawitz and Bordet & Delange were propounded by Nolf (1908) and Howell (1912).

Theory of Nolf

Nolf (1908) thought that five factors were required for normal blood coagulation, namely, thrombokinase (thromboplastin), calcium, thrombozyme, thrombogen, and fibrinogen. Thrombozyme and thrombogen, both constituting what was usually called "prothrombin", were activated by plasma thrombokinase, a lipoprotein having the same properties as tissue thrombokinase, to form thrombin. This, as in the

other theories, converted the fibrinogen to fibrin. Thrombozyme, he thought, was present in the platelets, leucocytes, vascular endothelial cells and the blood; 'Thrombogene', derived from the liver, was also circulating in the blood. Tissue cells, in his opinion, did not contain thrombozyme but accelerated coagulation in some non-specific way.

Theory of Howell

Howell (1910) considered that thromboplastin derived from tissues neutralised an antiprothrombin to release prothrombin. The latter, reacting with calcium, generated thrombin. Later he assumed that the newly discovered anticoagulant, heparin (Howell & Holt, 1918), was synonymous with his antiprothrombin. The platelets, he considered, supplied both thromboplastin and prothrombin; that they contained these factors was also suggested by Bayne-Jones (1912).

In all these theories, the platelets were believed to play a major role in the mechanism of blood coagulation.

Platelets and the Speed of Blood Coagulation

The role of platelets in blood coagulation was further clarified by the observation that when platelets are artificially removed from plasma by centrifuging or filtration, the clotting time of the platelet free plasma is considerably longer than that of platelet rich plasma (Sourd & Pagniez 1906; Cramer & Pringle 1913; Lee & Vincent 1914; Hess 1917; Tait & Greer 1926). The extraction of platelets from blood

by passage through a Berkefeld filter, may deprive the blood of more than platelets alone, and it is now known that such filtration will remove other coagulation factors and so prolong the clotting time. The use of silicone coated glassware (Jaques, Fidlar, Felsted & MacDonald 1946) greatly facilitated the removal of platelets from the blood in an undamaged state.by centrifugation. Patton, Ware & Seegers (1948), using this technique, were able to produce samples of dog plasma which, collected without anticoagulant and centrifuged at high speed, remained fluid for 72 hours. The coagulation time of human plasma in glass can also be prolonged by complete removal of the platelets (Brinkhous 1947). In order to achieve this prolongation of the clotting time, nearly all the platelets must be removed from the blood. The whole blood clotting time will remain constant over a wide range of platelet concentrations and is prolonged rarely in clinical thrombocytopenia (Quick, Shanberge & Stefanini 1949).

Platelets and Thrombin Generation

If platelets are merely reduced in number, and not removed completely, the clotting time of blood or plasma is not altered significantly from the normal. Thrombin generation is, however, markedly reduced and, as prothrombin is not consumed, abnormally large amounts of the latter are left in the serum after clotting is complete (Quick & Favre-Gilly 1949). This observation was confirmed by

Alexander & De Vries (1949), Conley, Hartman & Morse (1949), and Merskey (1950). Conley et al (1949) showed that even a fall in the platelet count from 350,000 to 260,000/cu.mm. decreased significantly the amount of prothrombin consumed during clotting.

Platelets and Thromboplastin

In the classical theory, as postulated by Morawitz (1905), thromboplastin or thrombokinase, released by the platelets or tissues, was considered to convert prothrombin directly. Only Nolf considered that thromboplastin might react with more than one factor before thrombin was generated from prothrombin. It was realised gradually that platelets had little direct thromboplastic activity as measured by comparison with tissue thromboplastin, (Quick 1947; Ware, Fahey & Seegers 1948;), and it became apparent that platelets or their thromboplastic component must act with some other factor or factors prior to activating prothrombin to thrombin. Howell (1912) first suggested that the thromboplastic component of platelets was related to cephalin and this observation was confirmed by Bordet (1920), Mills (1927), and Chargaff, Bancroft & Brown (1936). This work has assumed new importance with the isolation of the thromboplastic phospholipoid component from the platelets which has been identified as the fraction concerned in the conversion of prothrombin to thrombin (Creveld & Paulssen 1952;

Stefanini & Campbell 1954; Alkjaersig, Abe & Seegers 1955). A phosphatide (phospholipoid) component of soy bean has been shown to have equal activity (Chargaff et al 1936) and to correct the clotting defect in thrombocytopenic blood (White, Lagen, Aggeler & Geyer 1953). Similar material has been derived from human brain which can be substituted for platelets in 'in vitro' blood coagulation experiments (Bell & Alton 1954). Further, it has been claimed that the active fraction of these phospholipoids is phosphatidyl-ethamolamine. This has been shown to be capable of acting as a platelet substitute (Robinson & Poole 1956; Garrett 1956) and this fraction may also represent the 'thromboplastin' component of platelets (O'Brien 1956). There is considerable doubt, however, that this is so (Biggs & Bidwell 1957).

Other Coagulation Factors in Platelets

Ware, Fahey & Seegers (1948) demonstrated that platelets, as well as having thromboplastic activity, contained two other distinct factors which influenced blood coagulation, namely platelet factors 1 and 2. Both these factors were distinct from the thromboplastin component and were found in saline or water extracts of normal platelets (Creveld & Paulssen 1951).

Factor 1 appeared to accelerate the action of the thromboplastin on prothrombin to form thrombin. Factor 2 accelerated the rate at which thrombin would convert fibrinogen to fibrin. Platelet factor 3 was considered to

be the phospholipoid 'thromboplastin' component already discussed (Creveld & Paulssen 1952). These authors considered that factor 3 also had anti-heparin activity but this has been shown to be a property of a separate factor (Deutsch, Johnson & Seegers 1955). In addition Ware, Fahey & Seegers (1948) claimed that they found in water soluble platelet extracts a thrombin clottable fraction which apparently was not fibrinogen. This was called platelet factor 5 by Creveld (1954). It has still to be proved conclusively that this factor is not fibrinogen.

In addition to these factors, platelets have been shown to carry an anti-fibrinolysin (Johnson & Schneider 1953; Creveld 1954; Stefanini & Murphy 1956). 5-Hydroxytryptamine (Serotonin) is also contained in platelets and, while having marked vasoconstrictive properties and probably contributing indirectly to in vivo coagulation in haemostasis and thrombosis, it has not yet been shown to have any direct effect on the actual coagulation mechanism (Bounameaux & Lecomte 1954; Zucker & Borrelli 1955 a & b). It has been claimed that 5-H.T. is the principle responsible for clot retraction in bovine plasma (Fenichel & Seegers 1955) but this has not yet been shown to be the case in human blood (Magalini & Stefanini 1955).

Adenosine triphosphate (Born 1956), adrenaline and noradrenaline (Weil-Malherbe & Bone 1954), and glutamic oxalacetic transaminase (Magalini & Stefanini 1956) have also

been isolated from platelets but, so far, their influence, if any, on blood coagulation has not been determined.

Platelets and the Concept of Intrinsic Plasma Thromboplastin Formation

When blood is collected carefully without any tissue contamination, it will clot firmly in a glass tube. This fact suggested that the blood must have an intrinsic prothrombin converting mechanism independent of tissue thromboplastin. This intrinsic thromboplastin system takes 5 - 6 minutes to produce fibrin while tissue thromboplastin will act in 12 - 15 seconds. At first sight this suggested relatively little thromboplastic activity and the platelets were thought to provide what little thromboplastin appeared to be available. Quick (1947) and Brinkhous (1947) provided evidence to show that 'antihaemophilic globulin', a factor present in normal blood, but specifically reduced or absent in classical haemophilia, was essential for the coagulation promoting or prothrombin converting activity of platelets. As knowledge of blood coagulation developed, further factors were isolated which also appeared to react with platelets and augment their thromboplastic effect. Factor V (Owren 1947) was discovered during the investigation of a case of apparent congenital prothrombin deficiency in a woman of 29. The defect in this patient was, however, corrected by artificial prothrombin free plasma (adsorbed with aluminum hydroxide) as well as normal plasma. Owren showed that,

when isolated in a relatively pure state, factor V was essential for the conversion of prothrombin to thrombin by both tissue and platelet thromboplastin. That platelet factor 1 is in fact adsorbed factor V has been shown by Hjort, Rapaport & Owren (1955). Yet another prothrombin conversion factor was found in prothrombin-free serum (Owen & Bollman 1948) which was deficient in the blood of patients treated with 'coumarin' anticoagulants. This was named proconvertin by Owren (1951) or Factor VII (Koller, Loeliger & Duckert 1951). This latter name has been generally adopted in the British literature. Although factor VII was originally thought to be essential for the action of both platelet and tissue thromboplastin on prothrombin, it is now accepted that its sole function is the potentiation of the action of tissue thromboplastin (Koller 1955).

In 1952, Biggs, Douglas, Macfarlane, Dacie, Pitney, Merskey & O'Brien, and White, Aggeler, Glendening, Page, Leake & Bates independently discovered a further factor necessary for normal blood coagulation, which they called respectively Christmas Factor and Plasma Thromboplastin Component (PTC). It had become apparent that not all cases of classical haemophilia could be explained by a defect of antihaemophilic globulin and this new factor was found to be diminished or absent in these cases; the clinical syndrome being named Christmas disease by Biggs et al (1952).

Rosenthal, Dreskin & Rosenthal (1955) have defined a further factor which, when partially or totally absent from the blood, is associated with a mild haemophilia-like syndrome. This they named Plasma Thromboplastin Antecedent (PTA). This factor was shown to be distinct from AHG or Christmas factor.

Lastly, it has been claimed by Ratnoff & Colopy (1955), Frick & Hagen (1956) and Ramot, Singer, Heller & Zimmerman (1956) that yet another factor exists which is apparently essential for normal blood clotting and prothrombin consumption, and which has different properties to A.H.G., Christmas factor and Rosenthal's factor (PTA). When this factor is absent from the blood, there is no associated impairment of physiological haemostasis and patients with this defect are apparently normal. This has been called the 'Hageman' factor. The significance and the exact mode of action of these latter two factors is, as yet, obscure, but from the accumulated evidence all these factors would appear to react together with the platelets to form an intrinsic blood thromboplastin.

Originally, Biggs, Douglas & Macfarlane (1953b) showed that if platelets were incubated for several minutes with A.H.G., Factor V and Christmas factor, a thromboplastin was generated which was capable of clotting normal plasma in 8 - 10 seconds. Factor VII was thought originally to participate in this reaction but, since thromboplastin generation procedes normally in some cases in which Factor VII is absent (Koller

1955; Ackroyd 1956), it is, as already stated, thought to be necessary only for the action of tissue thromboplastin.

Biggs et al (1953b) used aluminium hydroxide adsorbed plasma to provide A.H.G. and factor V and serum to provide Christmas factor in their in vitro thromboplastin generating mixture and it is probable that these test components may also have contained the P.T.A. and Hageman factors now known to be essential for normal intrinsic thromboplastin generation.

The participation of five or more factors, in a reaction requiring several minutes to complete, suggested that they reacted in stages. While a defect of any one plasma or serum factor would prolong this phase and diminish the amount of thromboplastin generated, a deficiency in the number of platelets only diminished the amount of thromboplastin generated, but did not apparently prolong the interval before thromboplastin started to be formed (Biggs, Douglas & Macfarlane 1953 a & b).

Evidence was obtained to suggest that intermediate stages of thromboplastin formation could, in fact, be detected experimentally. It was thought that A.H.G., Christmas factor and platelets must first react to form an intermediate product which, in turn, reacted with factor V to form active intrinsic thromboplastin; calcium being required for all stages (Biggs et al 1953 a & b; Bergsagel 1956; Bergsagel & Hougie 1956).

Thus, it has been established beyond all reasonable doubt that platelets and their constituent factors are essential for normal intrinsic blood thromboplastin formation and normal blood coagulation.

Platelet Agglutination and Blood Coagulation

There has been general agreement that platelet disintegration plays an important part in the mechanism of blood coagulation. Stefanini (1953) thought that it constituted the first step and fundamental step in the entire process. While it was thought originally that the platelets, by disintegration, converted prothrombin to thrombin (Morawitz 1905; Bordet 1920) or directly released thrombin (Tait & Burke 1926), it is now known that their products of disintegration participate in the much more complex interaction with other factors before prothrombin is converted to thrombin (see p. 16).

While this has been accepted, it does not agree with the observation that white thrombi, made up of disintegrating platelets, form in damaged blood vessels without the clotting of the adjacent blood (Eberth & Schimmelbusch 1888; Burke & Tait 1926; Pickering 1928).

The formation of fibrin is usually the only visible change observed when blood clots but if platelet rich plasma freed from red cells is allowed to clot it is apparent that fibrin formation is preceded by sudden clumping of the platelets (Tait & Burke 1926). This phenomenon has been

recognised since the platelets were first discovered and was described by Hayem (1878), Bizzozero (1882) and Eberth & Schimmelbusch (1886). The latter authors coined the term viscous metamorphosis ('viscose metamorphose') to describe this change.

It is of historical interest to note that prior to this viscous metamorphosis of platelets had been observed by Osler (1874). He described the typical morphological changes observed in vitro and in vivo experiments, without appreciating their significance, and thought they might be pathognomonic of the 'exanthems'. Ranvier (1873) noted the presence of granular elements in the fibrin mesh work in clotted blood but thought they were a form of early fibrin.

Hayem (1878) noted that 'haematoblasts' or platelets became irregular, angled and etiolated during the process of blood coagulation, and fine delicate filaments projected from their surface. The platelets then formed masses which, in turn, became refractile, as the outline of individual platelets became more confused. Bizzozero (1882) described granular disintegration of the platelets as the only visible change in the formed elements of the blood when removed from the blood vessels and their observations led them to conclude that identical changes took place in vivo in the blood when a blood vessel was damaged. The platelets, normally quite separate in the blood stream, came together at the point of injury and formed a mass covering the damaged area and, as

small embolic clumps were broken off this mass by the blood stream, more platelets were added (Bizzozero 1882; Eberth & Schimmelbusch 1886). Hayem (1882) thought that fibrin eventually formed round these masses, but this was not confirmed by Eberth & Schimmelbusch (1888).

Hayem (1882) also showed that foreign material introduced in the blood vessels were covered rapidly with granular material consisting of clumped platelets.

The term 'viscous metamorphosis' (VM) was revived by Wright & Minot (1917) who, in a most extensive study of this phenomenon, showed that fresh serum was capable of inducing cell changes of VM in washed platelets derived from rabbits and humans. They also observed this phenomenon in clotting plasma. They reaffirmed the importance of calcium ions in this process thus confirming the previous observations of Mayer (1907), Cramer & Pringle (1913) and Zucker (1913). Their observations suggested that this process took place in two stages, namely agglutination of the platelets and 'viscous metamorphosis' proper. They thought that the former represented the finer grades of viscous metamorphosis. Their experiments were too detailed to review completely here but further reference will be made to them during discussion of the experimental work of this thesis.

Other authors have described the morphological changes of VM. Mayer (1907) called the large platelet clumps "forme de granules accoles sans structure." Aynaud (1911a) described the sequence of platelet

changes occurring during coagulation. Stübel (1914) clearly illustrated this process by a study of the platelets using darkground microscopy. Using the same method, Tait & Burke (1926) observed VM in 'native' platelet rich plasma, prepared without the addition of an anticoagulant, and noted the extrusion of granules by the platelets in this process. They thought that fibrin appeared along the pathway taken by these moving granules which they considered were composed of thrombin. Serial observations led them to conclude that there was a definite time relationship between the platelet changes and the formation of fibrin.

The presence of released gramules was confirmed by Ferguson (1934) who, however, considered that fibrin was formed in the free plasma and not in relation to the platelets or their granules. He also confirmed that calcium was necessary for this reaction. Further descriptions were given by Cramer & Bannerman (1929) and Tocantins (1938).

Platelet viscous metamorphosis has been studied by electron microscopy (Bessis & Burstein 1948; Bloom 1955; Alexsandrowicz, Blicharski & Feltynowski 1954; Braunsteiner, Fellinger & Pakesch 1954), and phase contrast microscopy (Bessis & Tabuis 1954; Bergsagel 1956; Setna & Rosenthal 1956).

Numerous attempts were made to define the factor or factors responsible for those changes.

Hayem (1882) considered that trauma instigated the mechanism of VM. Aynaud (1911a) postulated that tissue extracts contained extremely active platelet agglutinins or lysins. Roskam (1922) first suggested that the clumping of platelets was due to the flocculation of plasma proteins in "l'atmosphere plasmatique" around the platelets, and it was suggested that the material formed was fibrin (Apitz 1939). Fonio (1923 & 1940) was the first to suggest that thrombin might be capable of producing platelet viscous metamorphosis, probably converting fibrinogen to fibrin. This view has been widely accepted.

The theory that peri-platelet fibrin formation was responsible for VM was discarded when it was shown that, in congenital afibrinogenaemia, VM evolved normally (Pinniger, & Brunty 1946). Alexander, Goldstein, Rich, Le Bolloch, Diamond & Borges (1954) have recently confirmed this observation.

The interaction of antihaemophilic globulin and platelets in the formation of thromboplastin has already been discussed. Brinkhous (1947) considered that as A.H.G. required platelets before it could convert prothrombin to thrombin, it was the 'thrombocytolysin' responsible for platelet breakdown. Quick (1947) proposed the exactly opposite view and held that platelet activated 'thromboplastinogen' (A.H.G.) to thromboplastin.

Quick (1951) and Quick & Hussey (1952) renewed Fonio's hypothesis by postulating that, in the initial stages of blood coagulation, trace amounts of thrombin were generated, which, while being insufficient for the conversion of fibrinogen to fibrin, were capable of inducing VM in platelets. By virtue of this mechanism these authors considered that thrombin had an accumulative auto-catalytic action on the speed of blood coagulation. This hypothesis has received strong support from numerous workers who have observed the ability of thrombin to induce VM in platelets (Stefanini 1951 & 1953; Robertis, Paseyro & Reissig 1953; Desforges & Bigelow 1954; Zucker & Borrelli 1955c; Bounameaux 1955 & 1957b).

The latter three workers and Bergsagel (1956) did observe that human thrombin had no effect on washed platelets separated from their plasma. In addition, some of the previous claims were invalidated by Bergsagel's (1956) observation that, while human thrombin had no effect on washed platelets, bovine thrombin produced strong agglutination. He considered that this discrepancy was due, either to an interspecies reaction on the part of bovine thrombin, or to the presence of some impurity in bovine thrombin, such as an intermediate product of thromboplastin formation.

The recognition of the essential role of platelets or their products of disintegration in the generation of intrinsic plasma thromboplastin (Brinkhous 1947; Quick 1947; Biggs, Douglas & Macfarlane 1953 a & b; Bergsagel & Hougie

1956) led Bergsagel (1956) to suggest that a specific intermediate product of thromboplastin generation was formed by the interaction of Christmas factor, calcium and A.H.G., and that this was the factor responsible for inducing platelet VM and releasing a powerful coagulant. His experiments can, however, be criticised on the grounds that he demonstrated this phenomenon in a highly artificial coagulant mixture containing pig A.H.G., serum and calcium. The conditions existing in this were far removed from those of normal blood.

Contrary to all these previous opinions of the mechanism of platelet VM in blood coagulation, Mann, Hurn & Mathieson (1949) and Erkelens (1956) considered that no visible platelet change occurred until after fibrin had formed.

The influence of foreign or water wettable surfaces on platelets and their disintegration will be considered separately in Chapter VII.

Platelets and the Mechanism of Haemostasis

No discussion of the role of the blood platelets in the physiological mechanisms of the body and their relationship to blood coagulation, would be complete without some reference to the 'mechanism' of haemostasis.

It has already been shown how early workers believed that platelets played the most important role in haemostasis by blocking any breach in the vessel walls by virtue of their ability to undergo VM in the damaged segment and in the extravasated blood. Hayem (1882) showed that, if the

if he partially sectioned the jugular vein of the dog, the wound became filled with a granular mass of platelets surrounded by fibrin, and haemorrhage ceased. From this observation and those of Bizzozzero (1882) and Eberth & Schimmelbusch (1886), it was generally accepted that platelets together with fibrin formed a mechanical plug to stem the flow of blood from damaged vessels.

Macfarlane (1941) dissented from this accepted theory and pointed out that it failed to provide an acceptable explanation for the failure of haemostasis in many haemorrhagic diatheses. He stressed the role of the capillary in haemostasis and, from his experimental work, deduced that capillary contraction was an important factor in the control of haemorrhage. This contraction, as it passed off, was replaced by fibrin which formed in extravasated blood. The platelets, he relegated to a subsidiary role and considered their only function was to contribute their blood clotting components at the site of injury. His hypothesis was supported by the previous work of Lewis (1923) who had observed that active capillary contraction did occur and, in fact, was capable of exerting considerable pressure. Sanders, Ebert & Florey (1940) had also supported this contention by observing capillary contraction in rabbit ears following experimental trauma.

Marjorie Zucker (1947) restored interest in the role of platelets in haemostasis by observing haemostasis in the meso-appendix of the rat. She observed that when vessels

were sectioned completely they bled from both central and peripheral ends; gradually the stumps were occluded by masses of platelets which protruded into the wounds. In the arteries and large venules vaso-constriction occurred as the platelet plugs formed. Vaso-constriction also occurred in the adjoining undamaged vessels. If vaso-constriction passed off prematurely, bleeding recommenced around the platelet plug. She also observed that, while fibrin could not be demonstrated in relation to the platelet plug, the platelets in the latter fused to form an amorphous mass. These observations were confirmed by H.D. Zucker (1949) in a histological study of wounds in human skin. In damaged pre-capillary arterioles and venules, but not in capillaries, he found definite platelet masses, which had apparently undergone viscous metamorphosis, protruding into the wound. While no intravascular fibrin could be detected, it was observed in the extravasated blood in the wound and the walls of the wound track were lined by scattered clumps of platelets. In thrombocytopenic patients he found that fibrin alone, without platelets, appeared to be capable of producing adequate haemostasis. Platelet VM, he thought, commenced within 30 seconds of damage being inflicted on vessels. The role of platelets in haemostasis has been affirmed by Lutz (1951) and Fulton, Akers & Lutz (1953) in studies on the vessels in the hamster cheek pouch. These workers have filmed these changes and illustrated very clearly the striking intravascular platelet clumping when

vessels in the hamster cheek pouch are cut or damaged. (This film was shown in Oxford by Dr. Desai who had been associated with the above workers).

Chen & Tsai (1948) observing the haemostatic mechanism in the web of the frog's foot and in the rabbit, put forward yet another theory for vascular haemostasis. They observed contraction of capillaries in the frog but considered that pressure or cutting these vessels provoked adhesion between the lining endothelial cells. They stressed the importance of vascular contraction in arteries and arterioles but in venules platelet plugs appeared to play the more important role in stemming the flow of blood when the vessels were cut.

The ability of damaged vessels to contract had been observed by Magnus (1923) as well as M.B. Zucker (1947) and H.D. Zucker (1948). Janeway & Park (1912) and Janeway, Richardson & Park (1918) observed that serum contained a vaso-constrictive factor which did not appear to be epinephrine (adrenaline) and Tsai, McBride & Zucker (1944) and Zucker (1944) observed that this factor apparently originated in the platelets. The nature of this material was discovered by two sequences of coincidental research. Rapport, Green & Page (1948) and Rapport (1949) purified this serum constrictor and found that it contained equimolar parts of creatine, sulphuric acid, and indole derivatives. Tentatively they suggested it might be 5-hydroxytryptamine (5-H.T.) or 'serotonin'. Rand & Reid

(1951) confirmed that this 'serotonin' did come from platelets.

Almost simultaneously Erspamer and Asero (1952) demonstrated that 5-H.T. was the hormone secreted by the entero-chromaffin cells of the intestine. Rapidly these two observations were united and led to confirmation of the presence of 5-H.T. in platelets which had vaso-constrictive properties (Zucker, Friedman & Rapport 1954; Humphrey & Jaques 1954; Hardisty & Stacey 1955).

Humphrey & Toh (1954) showed that platelets took up 5-H.T. secreted by the entero-chromaffin system rather than forming it in situ.

Udenfriend & Weisbach (1954) considered that this 5-H.T. was released from the platelets when they were ruptured and the release of this hormone also accompanied physiological platelet VM (Zucker & Borrelli 1955b).

Thus it is now generally accepted that 5-H.T. or serotonin, being capable of contracting arteries and arterioles in its pure form, is the vaso-constrictive agent responsible for haemostatic vaso-constriction. Also it is probable that platelet VM releases 5-H.T. from the platelets (Zucker & Borrelli 1955b).

This discourse has shown haemostasis is a complex mechanism but the known features may be summarised as follows.

When blood vessels are damaged by trauma, haemorrhage may be stopped by:

1. the platelets undergoing viscous metamorphosis forming mechanical plugs and releasing agents responsible for

2. vaso-constriction of arteries, arterioles and venules;

3. the formation of fibrin;

4. capillary contraction either related to (2) or as a separate mechanism, and

5. endothelial adhesion in crushed vessels.

Whatever the role of fibrin it is apparently not essential for immediate or primary haemostasis but is necessary for permanent haemostasis.

All or some of these factors may operate in a given instance of trauma but it is probable that, while all may act synergistically in the normal individual, the failure of any one factor may be compensated by the increased activity of another. This problem must be viewed with caution, as it is by no means certain that the mechanism, as observed in experimental animals, is synonymous with that in humans. Neither has it been proven that the 'mechanism' of haemostasis in the superficial vessels of the dermis is identical to that in the deep tissues or viscera.

In some instances when the circulating blood platelets are reduced in numbers an abnormal haemorrhagic tendency ensues. This is usually characterised by a failure of the primary haemostatic mechanism as shown by a prolongation of the bleeding time. Also there is often abnormal fragility of the superficial capillaries of the skin.

As this failure of the haemostatic mechanism does not occur in every case of thrombocytopenia it must be assumed that, when it does occur, it is the result of at least two concomitant defects, one of the capillary walls, the other of the platelets themselves. The haemorrhage probably is the result of an escape of blood through the defect in the capillary wall and, the platelets being reduced in number or absent, the platelet plug does not form to repair or cover the defect. Furthermore it is reasonable to assume that where the platelets are reduced in number the vaso-constrictive agents released would also be decreased in amount or completely absent.

The abnormal bleeding in these thrombocytopenic syndromes will cease in any given site only as fibrin is formed to repair the breach in or around the affected vessel. As fibrin formation is not so dependent on platelet numbers, fibrin will form in nearly normal time in spite of severe thrombocytopenia. If platelets are completely absent from the peripheral blood, this mechanism may also fail, but it is probable that a complete absence of circulating platelets occurs only in exceptional cases such as true bone marrow aplasia or terminal acute leukaemia. In these examples a much more severe and intractable form of spontaneous haemorrhage is encountered.

If the blood platelets are present in normal numbers, but have abnormal function, a similar failure of the primary haemostatic mechanism may be found.

A congenital or acquired defect of one or more of the plasma or serum coagulation factors necessary for intrinsic thromboplastin formation is often accompanied by a more severe haemorrhagic tendency than that encountered in the thrombocytopenic syndromes. Unlike the latter, there is seldom a defect in the mechanism of primary haemostasis. Thus, while trauma does provoke a normal blood loss, this stops in normal time only to restart after a variable period. It must be presumed, in these cases, that the platelet plug and vaso-constrictive mechanisms function normally but, as imperfect or no fibrin is formed at the site of the trauma, its reinforcing action fails and so secondary haemorrhage occurs. The intractable nature of this secondary haemorrhage, such as is seen following trauma in cases of haemophilia, suggests that, once the primary haemostatic mechanism has passed off, it cannot reappear at the primary site unless fresh trauma occurs.

While this does explain the type of haemorrhage in certain syndromes, anomalies do exist which are encountered too frequently to be ignored.

In some cases of severe haemophilia there is a failure of the primary haemostatic mechanism as measured by the bleeding time, in spite of the presence of a normal number of normally functioning platelets. Also in some examples of athrombocytopenic purpura, a failure of primary haemostasis is associated with a normal number of apparently normal functioning platelets and a low level of antihaemophilic

globulin. In spite of the latter, these cases do not usually show severe secondary haemorrhage. Further, severe and intractable spontaneous haemorrhage can occur from dilated capillaries in hereditary telangiectasia where platelet numbers and function and coagulation are all entirely normal. In such cases the primary mechanism of haemostasis only fails when an abnormal vessel is damaged, but once started, blood loss will continue in spite of apparent normal fibrin formation in the shed blood. This does stress that the role of capillary contraction in haemostasis requires further investigation.

The overwhelming difficulty in research into this problem has been the inability to observe blood vessels in vivo in other than experimental animals. In the human the only vessels that can be visualised are the capillaries in the nail bed, the skin, and the conjunctival and retinal membranes of the eye. Also it appears to be impossible, at present, to definitely differentiate fibrin from platelets by any orthodox histological means. Until these problems are solved, this subject will continue to be one of controversy.

This review has showed that platelets play an essential part, together with other plasma coagulation factors, in the initial sequence of blood coagulation and thromboplastin formation, as well as in the mechanism of haemostasis. But it was not at all clear exactly when or where the platelets

or their disintegration products acted in the sequence of blood coagulation and what factor or factors controlled the evolution of viscous metamorphosis. As it was essential that these mechanisms be more fully understood before any attempt could be made to determine whether agglutinating factors, other than those existing in the coagulating mechanism, were present in the blood of patients with idiopathic or other forms of thrombocytopenic purpura, it appeared to be worth while to re-examine these problems in the light of modern knowledge.

CHAPTER II

Viscous Metamorphosis and Platelet Agglutination

When whole blood is allowed to clot in vitro the only visible change is the formation of fibrin.

If the red cells are removed, and platelet rich plasma is observed as it clots, fibrin formation is preceded by sudden and obvious clumping of the platelets. This change is called "viscous metamorphosis" by some workers or "platelet agglutination" by others. There is confusion in the existing literature as to the exact definition of these terms and it will be stressed in the course of this thesis that they are not synonymous, but that one may easily be mistaken for the other.

In the experimental work to be described the term "viscous metamorphosis" (VM) will be used to include all the characteristic morphological changes that platelets undergo during the process of blood coagulation. The term "platelet agglutination" will be employed to describe the simple clumping of unaltered platelets, a process morphologically distinct from viscous metamorphosis.

To compare these two phenomena, platelet changes were first observed in clotting normal platelet rich native plasma. The separation of platelets in such plasma was achieved by cooling the collected blood in siliconed tubes

contained in melting ice in a small thermos flask. The red cells were removed by low-speed centrifuging at 0°C (see Appendix A, p. 11). By this technique it was possible to obtain platelet rich plasma, free from anticoagulant, which did not clot for several hours if kept at 0°C. Samples of the plasma were placed in glass tubes and allowed to clot by warming to 37°C; the tube being agitated to allow the platelets to collide.

Normal Viscous Metamorphosis (Phase Contrast Microscopy)

As the platelet rich native plasma was warmed to 37°C the platelets could be observed forming microscopic clumps which gradually increased in size until they were eventually enmeshed in fibrin as it formed. Attempts to observe this sequence taking place in microscopic preparations were unsuccessful as the pressure of the coverslip, necessary for good phase-contrast microscopy, prevented the platelets colliding with each other, and thus their ability to undergo typical VM. Therefore serial samples both fixed and unfixed, taken from the tubes as the platelet rich plasma clotted, were examined by phase-contrast microscopy. The first visible change involving individual platelets took place at the periphery of these cells, where pseudopodia, in the form of filaments, developed. As the platelets started to clump, they were joined together by union of these filaments, and, as they did so, the size of the individual platelets increased.

The clumps further increased in size to form granular masses of platelets in which the majority of platelets had lost their individual outlines (Figs. 1 & 2). A striking feature of this change was the tenacious and irreversible union of one platelet with the other by means of hyaline bands, so that one platelet merged imperceptibly with its neighbour (Figs. 3 & 4). These illustrations do suggest that these platelet clumps were flat and pancake-like, but this appearance was induced by pressure on the coverslip prior to photomicrography and does not represent the true sequence of morphological changes. In fact, the platelet clumps were irregular spherical masses, a state impossible to illustrate satisfactorily by phase contrast microphotography. In the early stages of VM only some of the platelets were incorporated into these masses and numerous individual platelets could be seen lying free in the plasma. Gradually the latter either adhered to already existing clumps or formed fresh clumps.

Once these large granular masses had formed, the platelets in their centres started to lyse and change into amorphous structureless material (Figs. 5 & 6). In parallel with these changes, granular material, apparently originating from the periphery of the platelet masses, appeared suddenly in the plasma (Fig. 7). As VM developed more and more of these granules appeared until, just prior to fibrin formation, the plasma contained numerous small granules undergoing violent Brownian movement. Further, it was

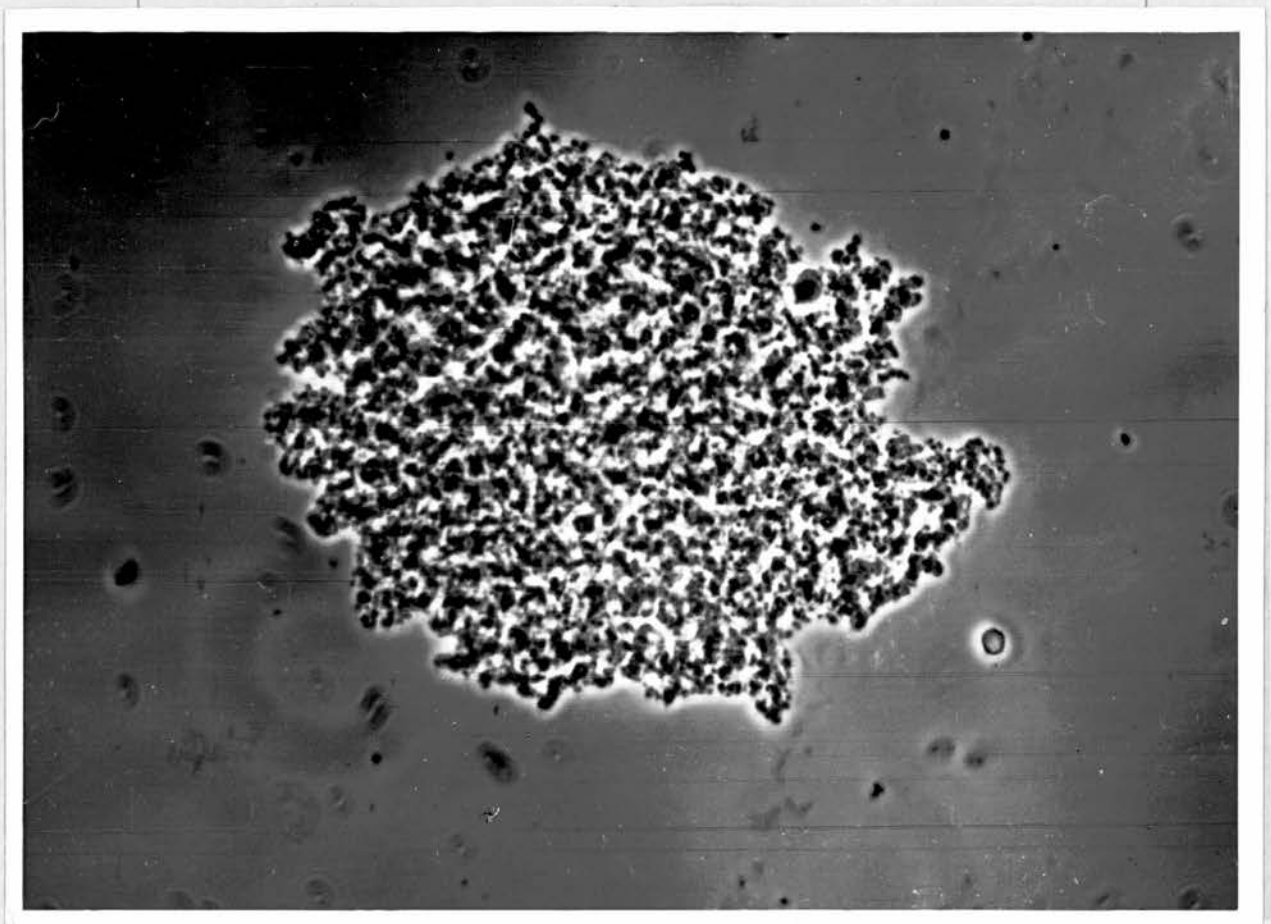

Figure 1
(Phase contrast x 1100)

Early Viscous Metamorphosis

The typical granular mass of platelets formed in the early stages of VM.

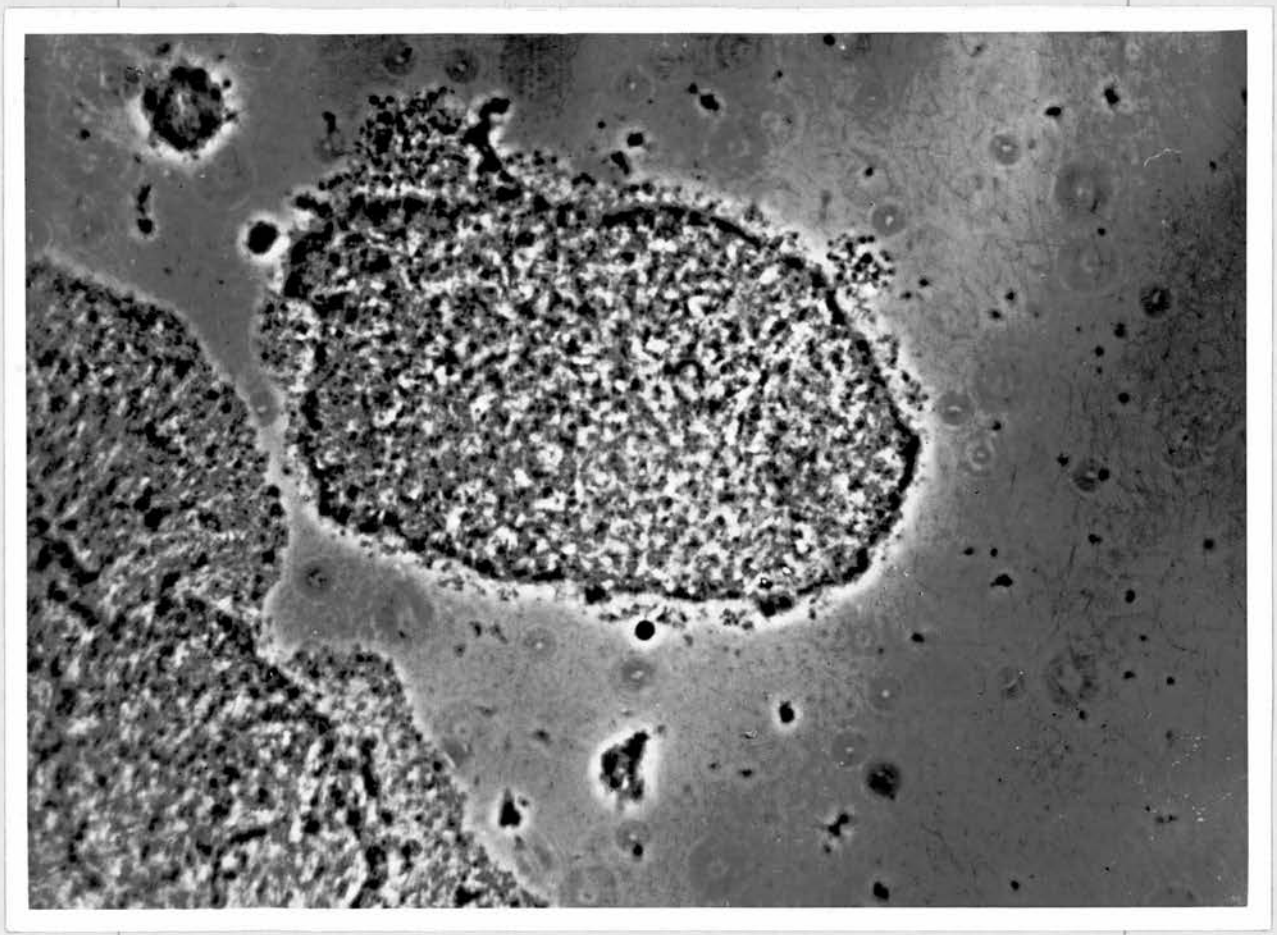

Figure 2
(Phase contrast x 1100)

Viscous Metamorphosis

A granular mass of fused platelets in which the individual platelets have commenced to merge with one another.

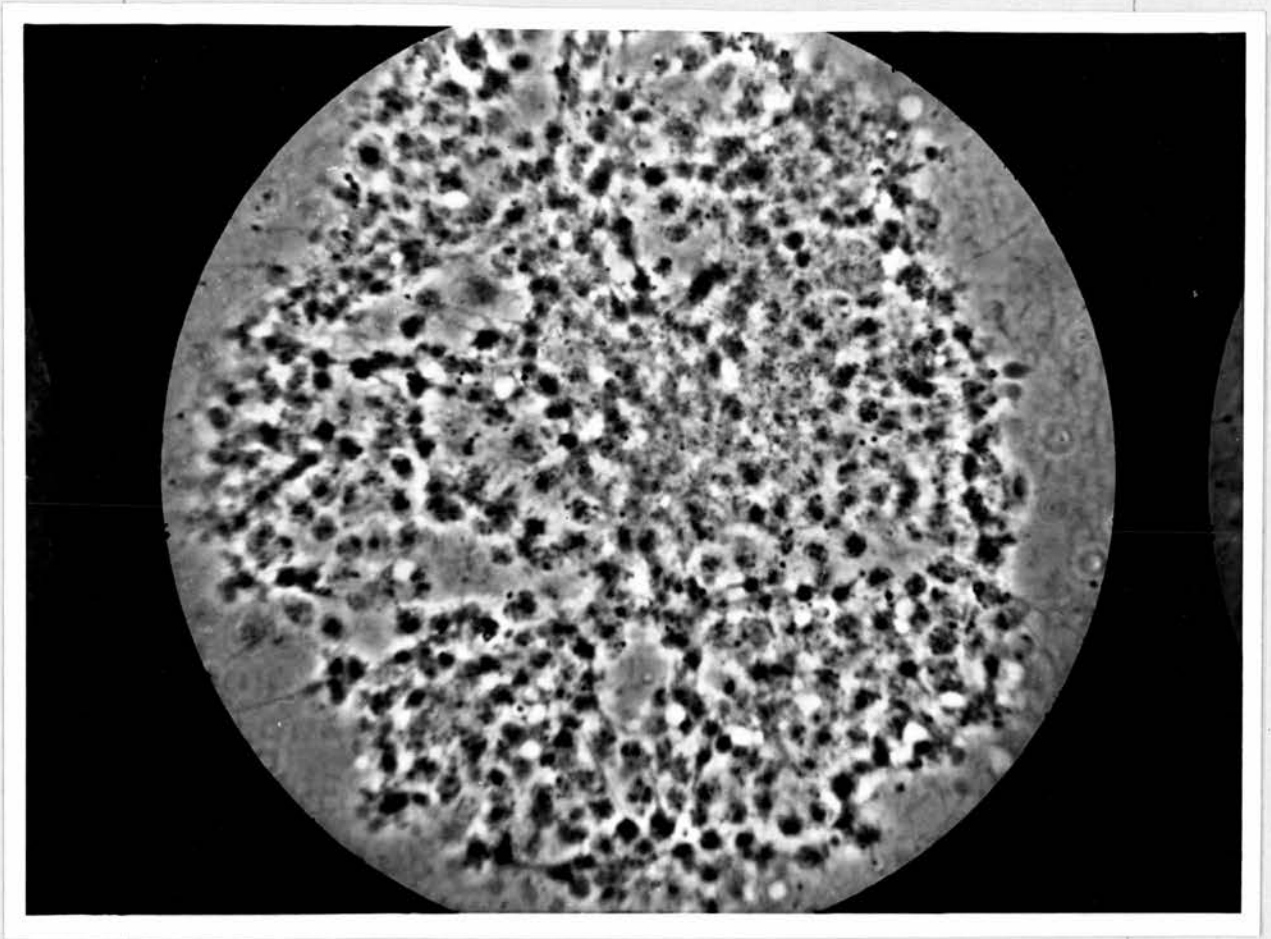

Figure 3
(Phase contrast x 5500)

Figures 3 & 4

Viscous Metamorphosis

A higher magnification of the platelet clump showing that the platelets adhere to one another by means of hyaline filaments. The platelets in the centre of the clumps have begun to lyse.

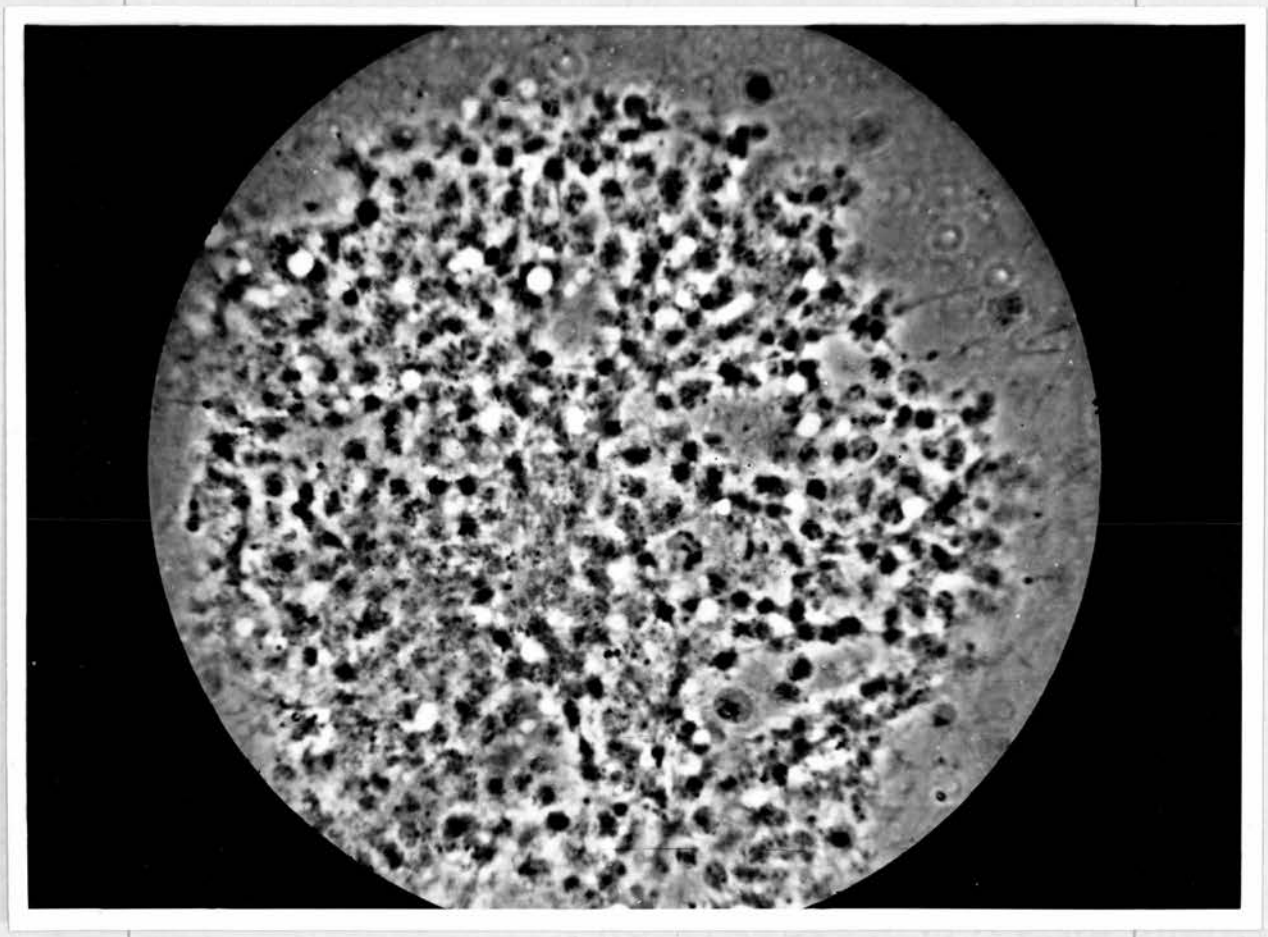

Figure 4
(Phase contrast x 5500)

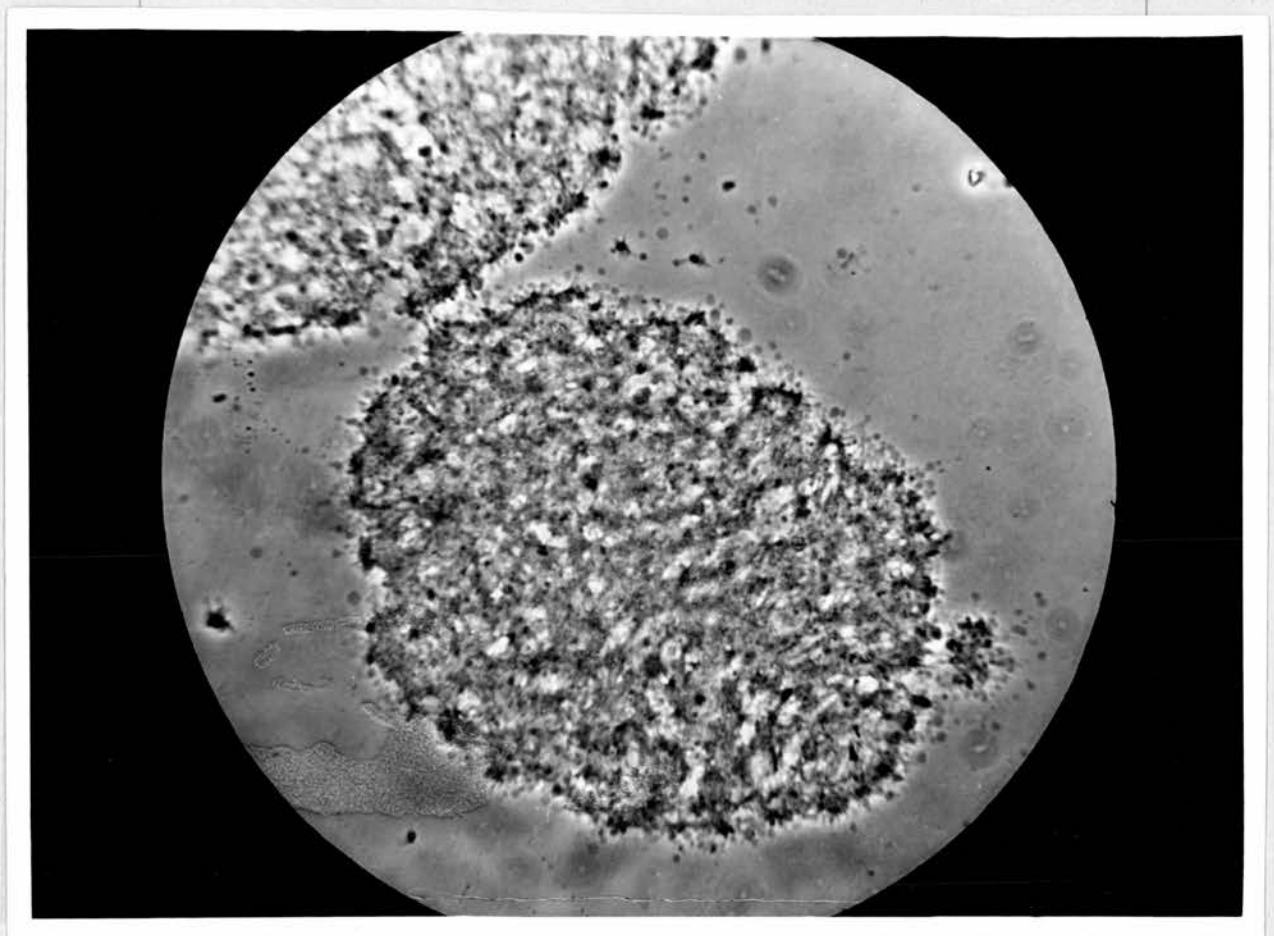

Figure 5
(Phase contrast x 1100)

Figures 5 & 6

Viscous Metamorphosis

More advanced platelet lysis and the formation of amorphous debris in the centre of each clump.

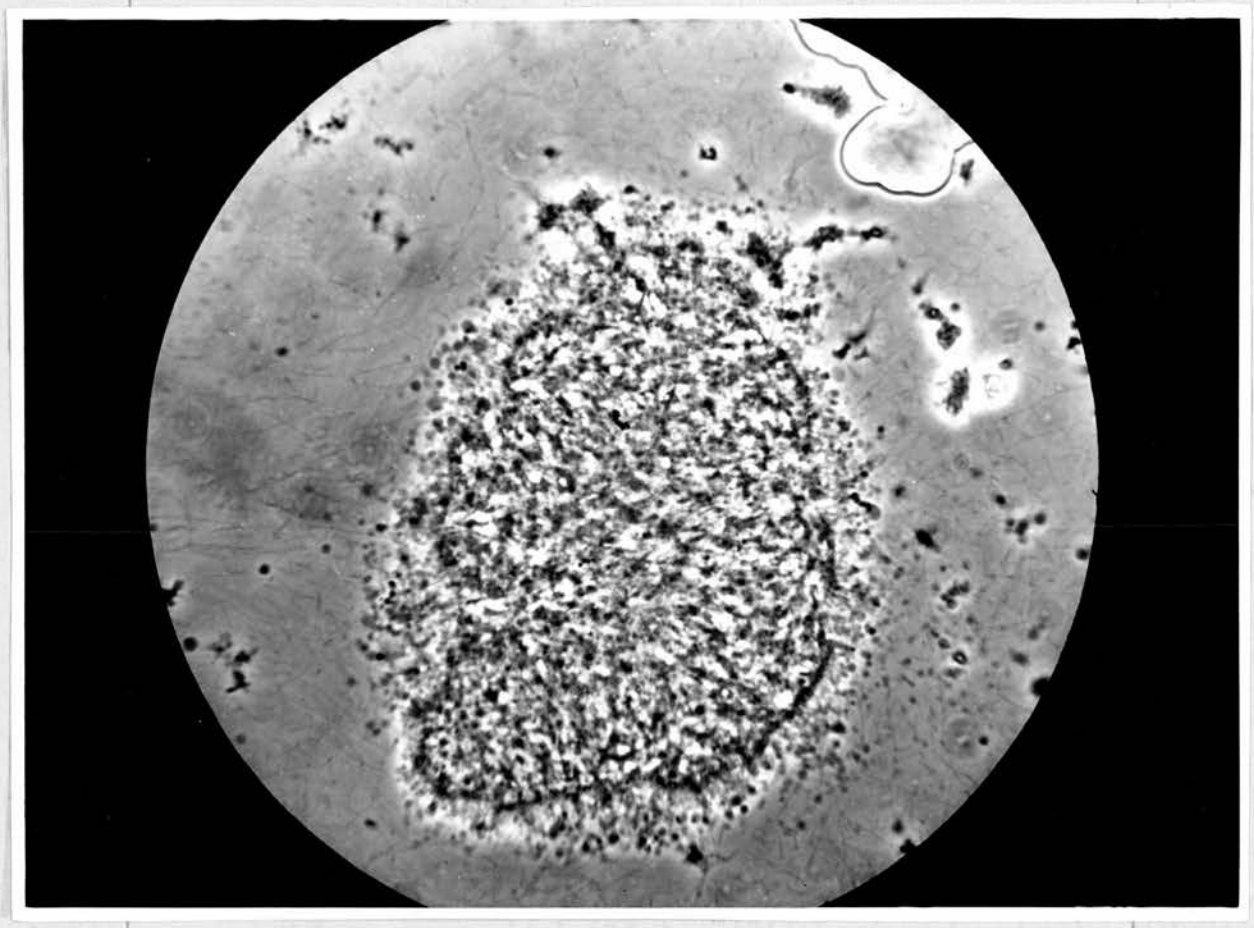

Figure 6

Phase contrast x 1100)

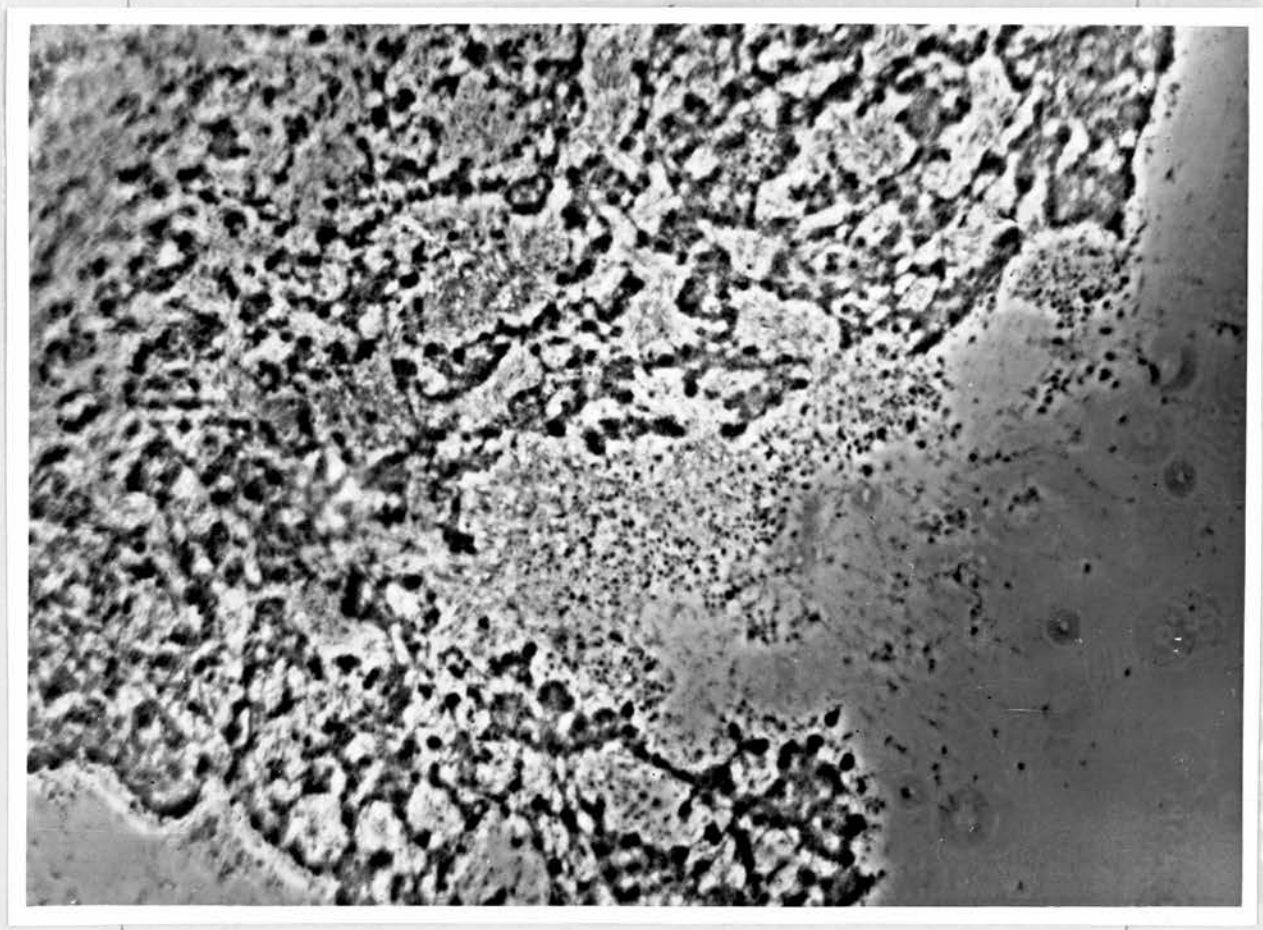

Figure 7
(Phase contrast x 2200)

Viscous Metamorphosis

Platelet granules being released from the periphery of the platelet clump.

evident that some factor limited the size of the platelet masses as, once the latter had reached a certain size, they did not adhere to each other or unclumped platelets. A typical example of a clump which had reached maximum size is illustrated in Fig. 8.

Fibrin was seen to form first in the intervening plasma and in close relationship to the released granules. Appearing first as small crystal-like rosettes of fibres, the fibres rapidly joined the platelet masses together by adhering to the periphery of the clumps (Fig. 8).

Once fibrin had formed, the metamorphosed platelets underwent yet further changes. Around the periphery of the clumps small clear white 'balloons' formed, which pushed their way slowly outwards between the fibres of fibrin. Some eventually became detached and were seen to float free from the platelet clumps (Figs. 8, 9 & 10). This change was not fully developed until approximately 30 minutes after fibrin had formed. These balloons also formed in the centre of the platelet masses (Fig. 11).

The changes that have been described are thought to be typical of "viscous metamorphosis" and are entirely different from the sequence of "platelet agglutination".

Platelet Agglutination (Phase Contrast Microscopy)

As this property of platelets will be studied in the experimental work of this thesis, it is convenient to

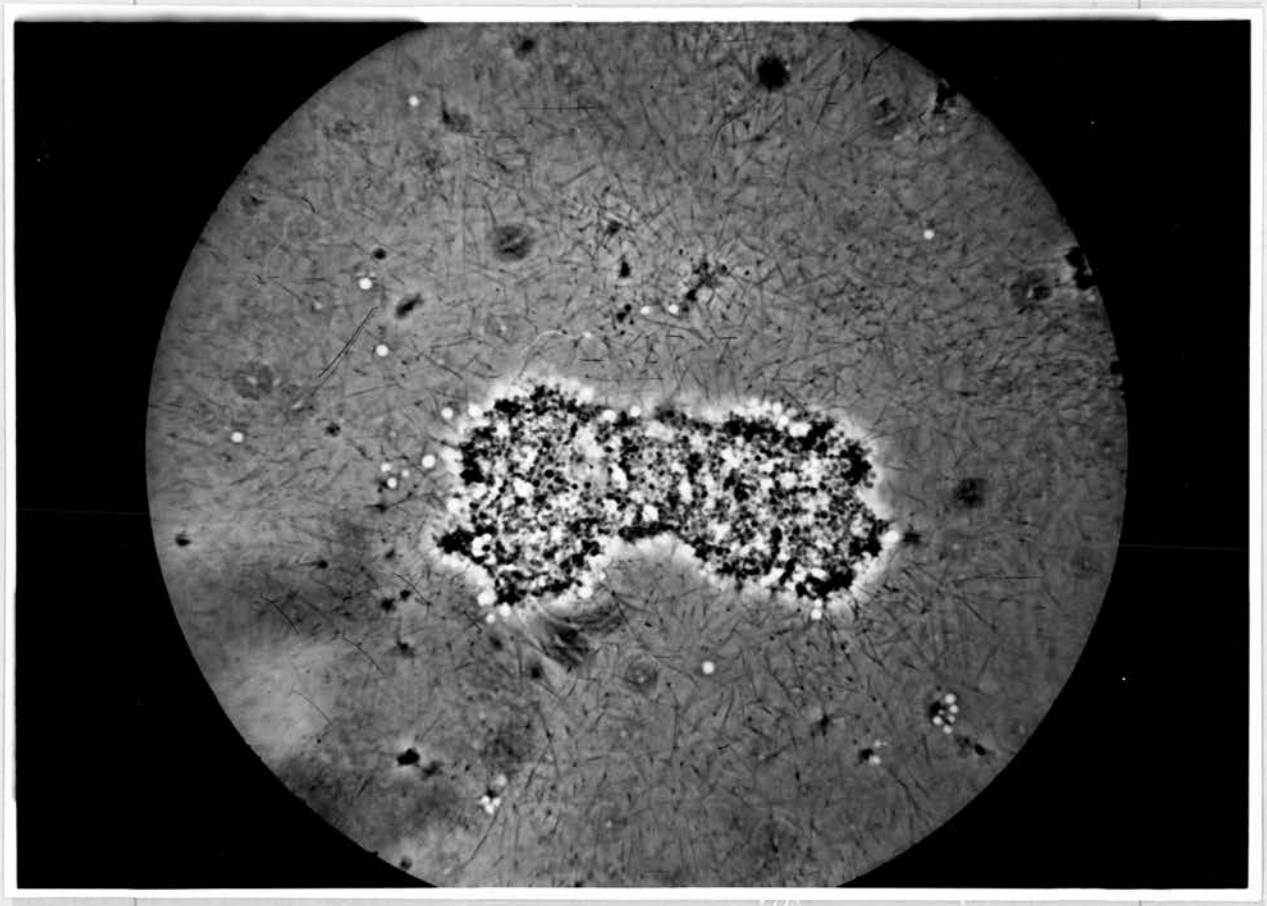

Figure 8
(Phase contrast x 1100)

Late Viscous Metamorphosis

A typical platelet clump in plasma showing the distribution of fibrin fibres. Small white 'balloons' are present around the periphery of the clump and some have escaped into the surrounding plasma (native plasma).

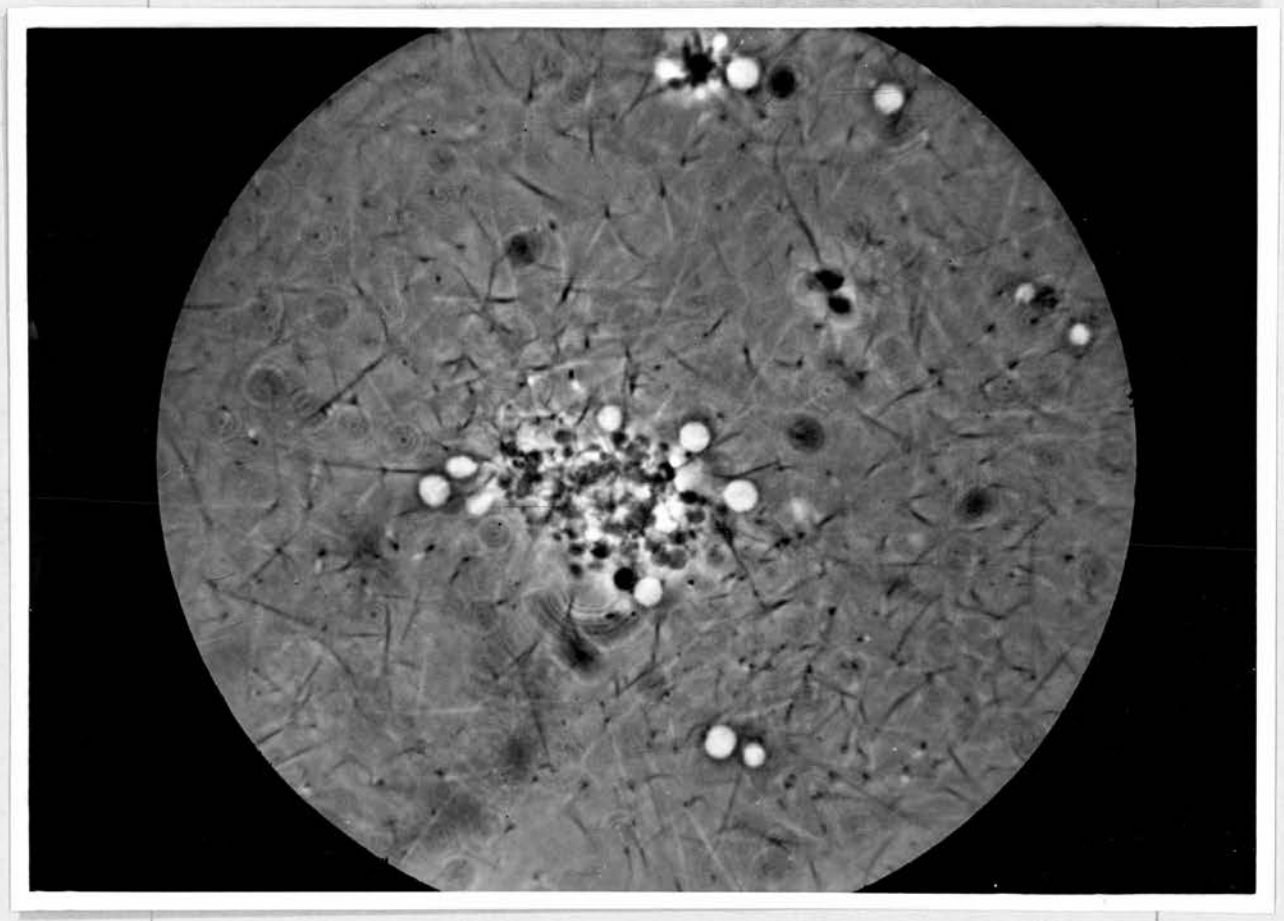

Figure 9
(Phase Contrast x 5500)

Figures 9 & 10

Late Viscous Metamorphosis

The formation of white 'balloons' at the periphery of the platelet clump. Each 'balloon' is extruded between the adhering fibres of fibrin. Some 'balloons' are lying apart from the clump. (Native plasma).

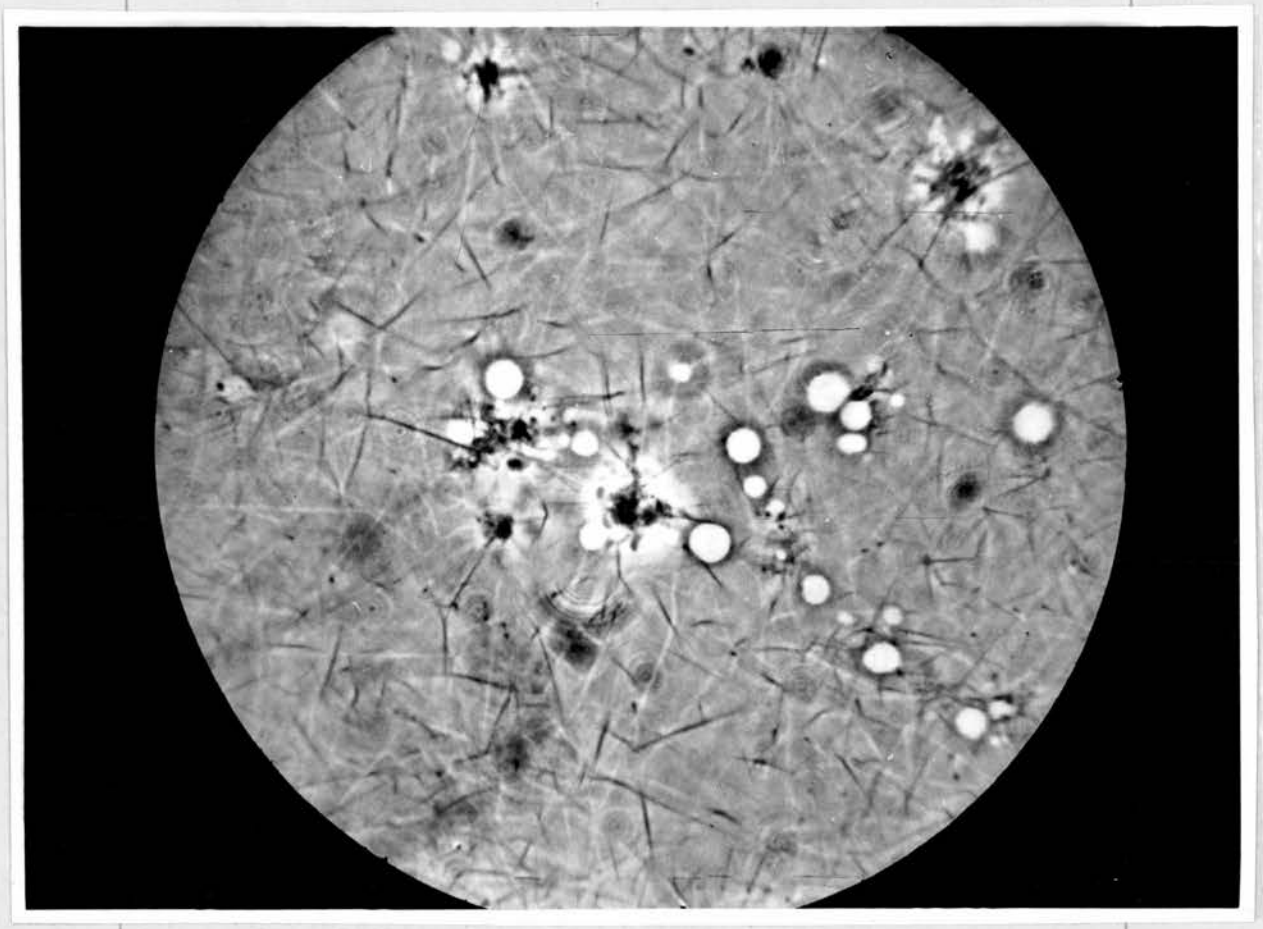

Figure 10

(Phase Contrast x 5500)

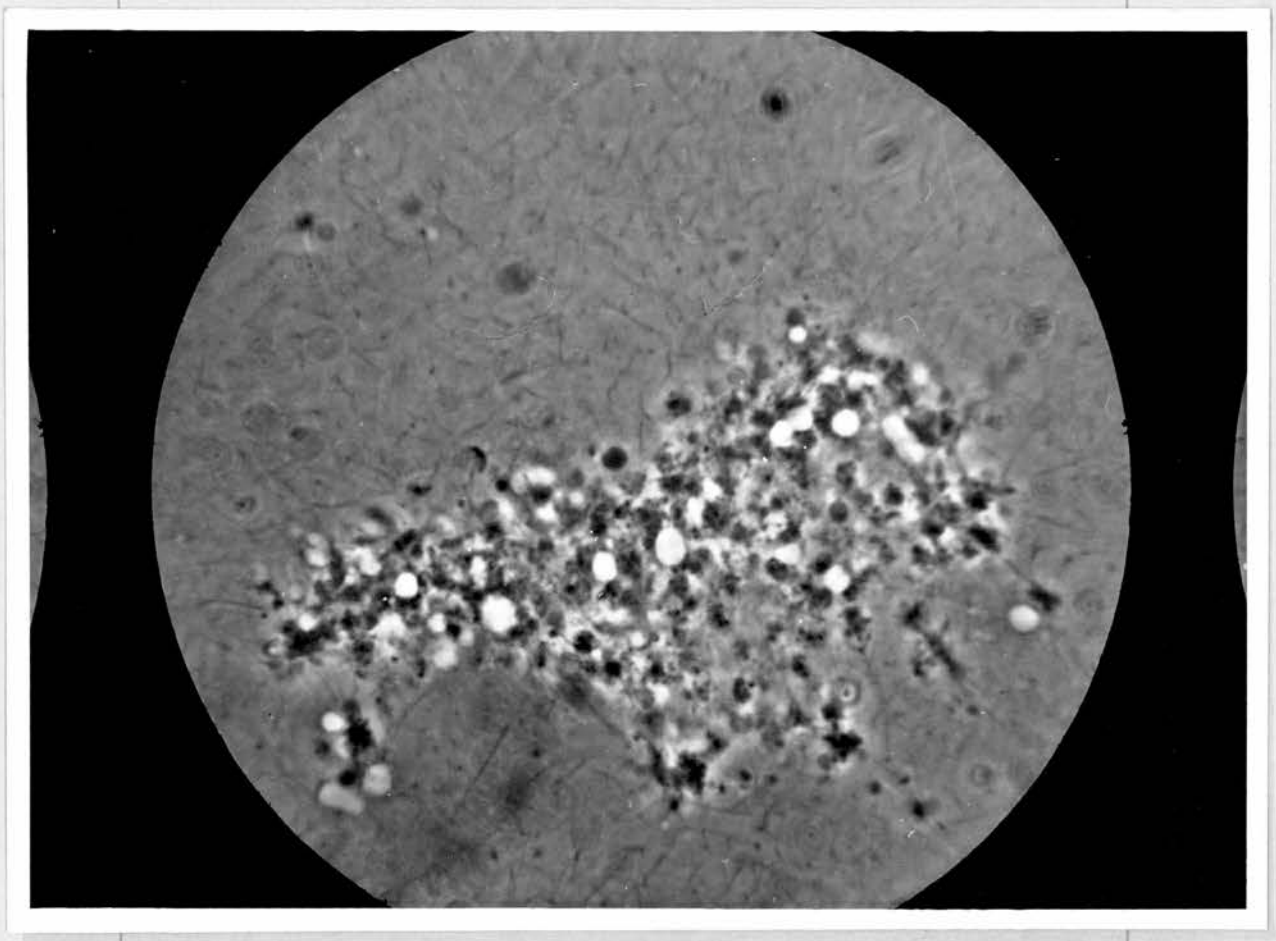

Figure 11
(Phase contrast x 5500)

Late Viscous Metamorphosis

The formation of white balloons in the centre of the platelet mass. (Native plasma)

describe the typical morphological appearances along with those of viscous metamorphosis.

Platelet agglutination was induced in vitro by anti-human platelet rabbit serum (Appendix B, p. 31). After suitable treatment to remove those coagulation factors that might induce VM (see Chapter 5 and "Platelet Agglutination Test" Appendix A, p.14), the sera were added to washed human platelet suspensions and the morphological changes observed during and after 1 hour's incubation of the mixtures at room temperature.

Within fifteen minutes of the antiserum being added to the platelets the latter formed small clumps or agglutinates (Figs. 12 & 13). In these the individual platelets were easily distinguishable and, unlike VM, all the platelets had agglutinated, first into small clumps of two or three platelets, and then by these uniting, into larger clumps; no free unagglutinated platelets could be seen. The platelet clumps did not appear to form irregular spheres and, once agglutinated, no other morphological changes took place even after 2 hour's incubation. After standing for several hours at room temperature some swelling and lysis of individual platelets was observed. These changes, however, were also seen in platelet suspensions, to which no agglutinating sera had been added, after storage for similar periods.

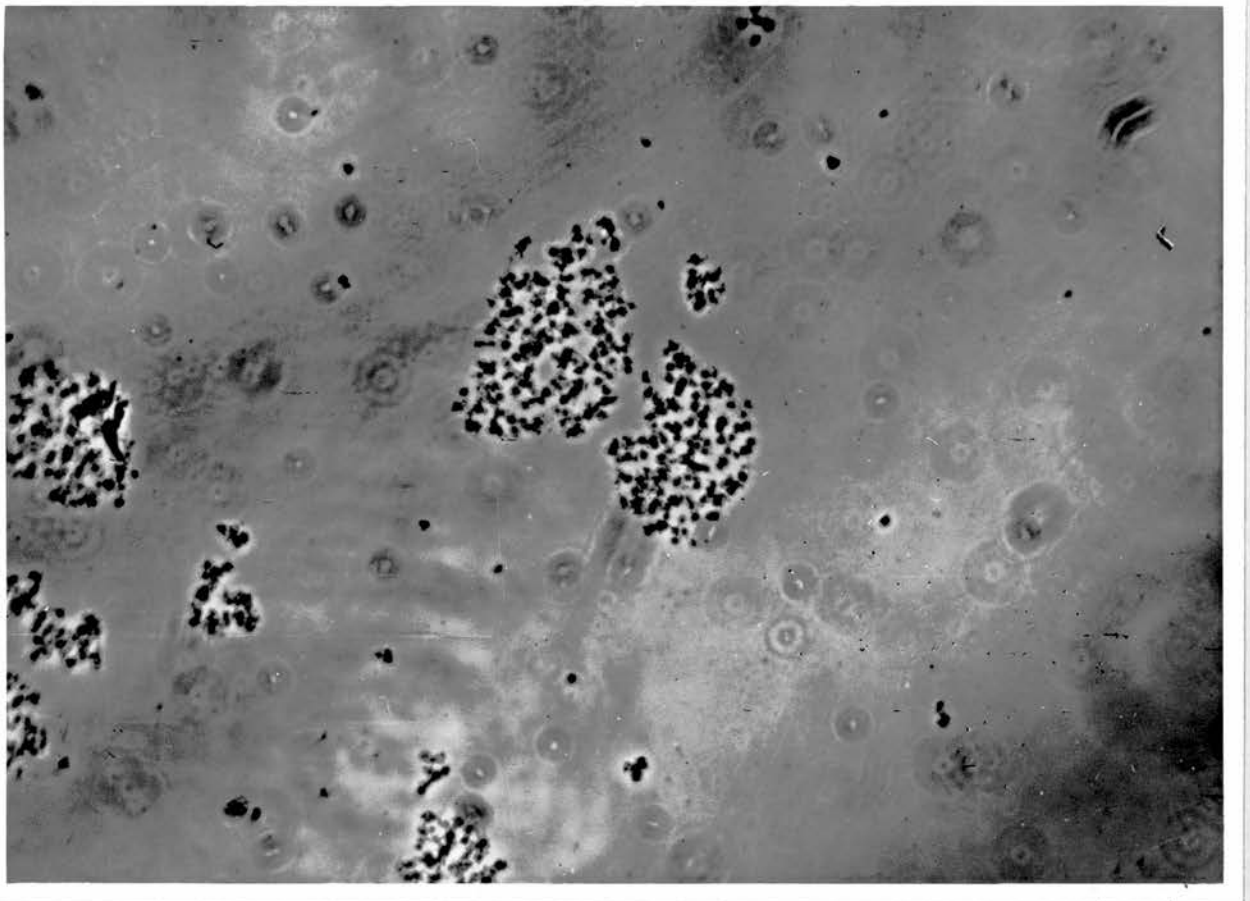

Figure 12
(Phase Contrast x 1100)

Figures 12 & 13

Immune Platelet Agglutination

The platelets have retained their individuality and no lysis or fusion has taken place.

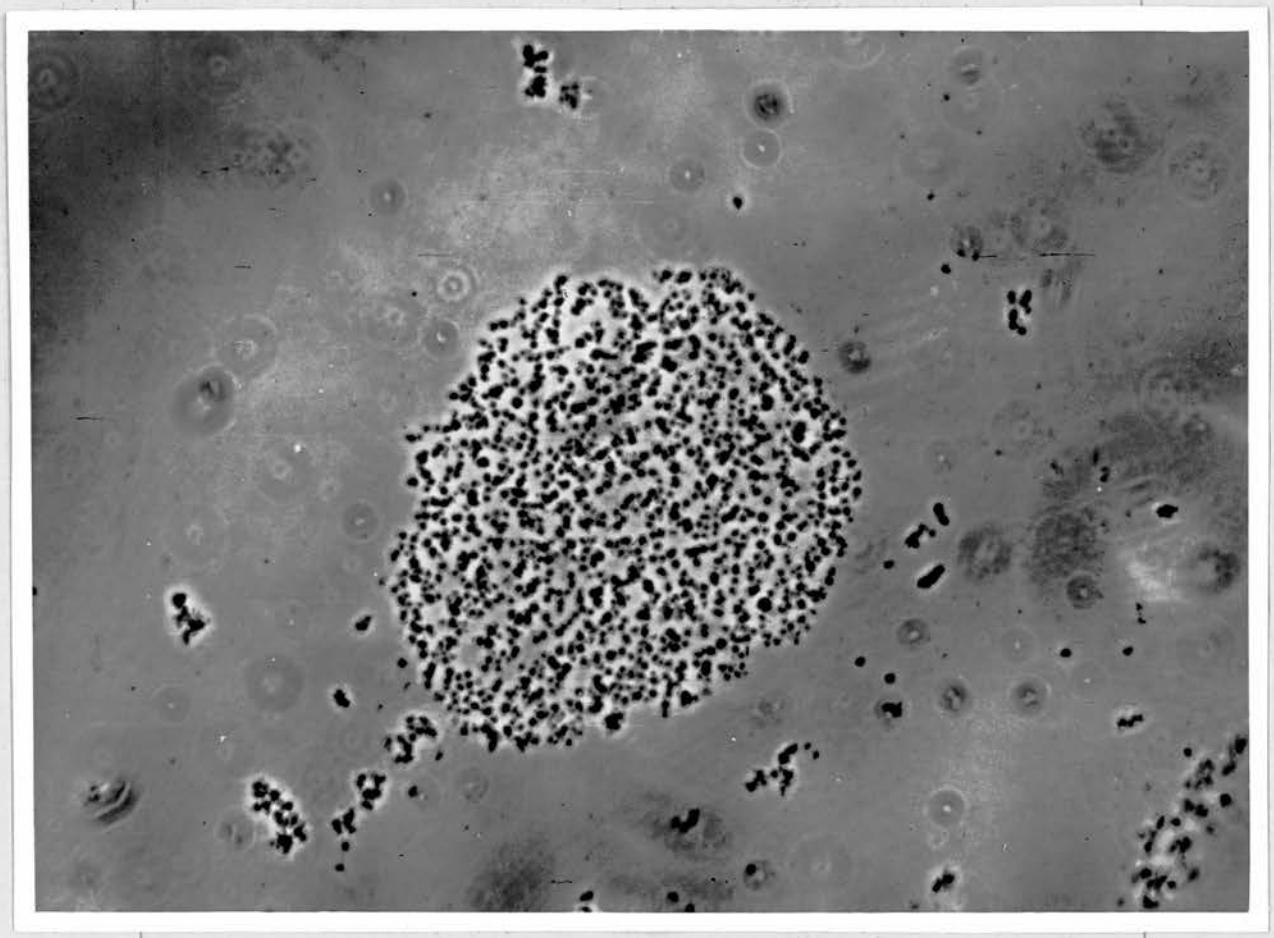

Figure 13

(Phase Contrast x 1100)

CHAPTER III

Platelet Viscous Metamorphosis and its Relationship to Fibrin Formation

While the morphological changes of platelet viscous metamorphosis (VM) have attracted the attention of numerous workers, very little attention has been paid to direct observation of this phenomenon in relation to fibrin formation. Aynaud (1911a) called the onset of VM "le premier temps de coagulation" and Tait & Burke (1926) suggested that VM did bear time relationship to fibrin formation. Personal observation of normal platelet rich plasma as it clotted, suggested that this latter conclusion was correct. A study of this phenomenon in abnormal plasma was thought to be likely to provide information of importance. Therefore platelet VM was observed in both normal plasma and in plasma derived from patients in whom the existence of congenital or acquired defects of essential coagulation factors had been established. Platelet rich native plasma was used in these experiments (Appendix A, p. 11 Experiment 1, Appendix B, p. 1)

Normal Platelet Rich Plasma

The evolution of VM was studied in 32 normal people. In all, VM followed the typical sequence already described. The time of onset of the first visible platelet clumping and fibrin formation were recorded (Table 1).

TABLE I Experiment 1 Appendix B, p.1

The relationship of viscous metamorphosis to fibrin formation in normal and abnormal plasma.

0.5 ml. platelet rich native plasma was allowed to clot at 37°C and the onset of viscous metamorphosis and fibrin formation recorded.

Diagnosis	No.of Obs.	Start of Platelet Clumping Mean in Secs.	Range of Obs. in secs.	Clotting Time. Mean in Secs.	Range of Obs. in secs.	Interval Mean in Secs.	Range of Obs. in secs.
NORMAL	32	47.8	10 - 165	214	135-290	160	80 - 226
HAEMOPHILIA							
Mild (0-400 secs)	5	26	15 - 95	311	255-376	285	238-359
Intermediate (400-700)	11	58	15 - 90	552	450-690	494	432-687
Severe (700+)	2	35	25 - 45	1325	870-1780	1253	825-1755
CHRISTMAS DISEASE							
Mild (0-400)	5	24	15 - 45	322	257-405	298	240-390
Moderate (400-700)	1	12	-	547	-	535	-
Severe (700+)	1	10	-	2420	-	2410	-
DINDEVAN TREATED CASES							
Moderate defect (50 - 10%)	1	20	-	230	-	210	-
Severe defect (10-00%)	3	33.6	30 - 40	501	465-542	468	435-511
VON WILLEBRAND'S DISEASE	5	40	25 - 55	323	225-395	283	185-346
HEPARIN 30 mins. after 5000 u. I.V.	1	66	-	1061	-	995	-
FACTOR V	1	25	-	485	-	460	-
CIRCULATING ANTICOAGULANTS	1	20	-	2985	-	2965	-
THROMBOCYTOPENIA	2	--	-	405	-	-	-
		--	-	456	-	-	-
ROSENTHAL'S SYNDROME (P.T.A. Deficiency)	3	421	295-550	527	357-640	106	32 - 203

These observations were made either by observing these changes in the tubes by naked eye or, alternatively, by taking serial samples at 30 second intervals in order to observe the morphological changes by phase contrast microscopy (Appendix A, p. 8). By this method it was evident that a definite time sequence evolved between the onset of the platelet clumping and fibrin formation. While the interval between the start of VM and fibrin formation was relatively constant the time of onset varied considerably (Table 1) and this was also true for the speed at which this change evolved.

Haemophilic Plasma (Table 1)

Platelet rich native plasma was obtained from patients with varying degrees of severity of haemophilia. 15 individual samples were examined and, in all, VM began normally and in the normal time. Fibrin formation was delayed in every instance, according to coagulation time of the plasma and the interval between VM and fibrin formation was significantly greater than in normals (Table 1).

In platelet rich plasma from those patients with moderate or severe haemophilia, the platelet clumps once formed began to break up and disappear before fibrin formed, only to reappear within seconds of fibrin formation. Some of this disappearance was due to adhesion of occasional clumps to the glass, but microscopic observation of the plasma confirmed that the majority of platelet clumps had partially broken up.

Christmas Disease

Seven individual samples were examined from patients with diminished or absent Christmas factor (Table 1). In all, VM showed a sequence and intensity identical to the normal process. As in the haemophilic samples, VM and fibrin formation were dissociated to a significant degree.

Phenylinanedione ("Dindevan") Treated Cases

Four cases receiving therapeutic doses of Dindevan (Phenylinanedione) were examined (Table 1). The blood from such cases is deficient in Factor VII (Walker & Hunter 1954). All showed normal VM. One example, with an induced defect of 22% as measured by the Proconvertin and Prothrombin technique (Owren & Aas 1951) showed no significant variation from the normal range. Three cases with a severe defect (0-10%) showed significant dissociation between VM and fibrin formation.

Heparinised Normal Plasma

Blood was examined 30 minutes after the intravenous administration of 5,000 units heparin into a normal person (Table 1). VM took place normally but, as in cases of moderate or severe haemophilia, the tenacity of the clumps was impaired and they tended to break up before fibrin formed. Again the interval between VM and fibrin formation was prolonged.

Factor V Deficiency

Platelet rich native plasma from a patient with a marked

congenital deficiency in Factor V was observed as it clotted. VM occurred normally within 1 minute, but fibrin formation was delayed and a definite dissociation between VM and fibrin formation existed (Table 1).

Circulating Anticoagulant

One patient was examined in whom a high level of circulating anticoagulant inhibited the formation of thromboplastin (probably by direct action on antihaemophilic globulin). In this case the platelets underwent VM in normal time (Table 1) but, as in haemophilia and heparinised blood, the clumps tended to break up and, after 10 minutes had elapsed, had virtually disappeared. Fibrin formation was delayed and, although fibrin appeared in 45 minutes, it was still forming at 90 minutes.

Thrombocytopenia

In plasma prepared from two cases of idiopathic thrombocytopenia (21,000 and 34,000 platelets/cu.mm.) no platelet VM could be detected. Microscopic examination did however show morphological changes in individual platelets and granules were released into the plasma. The latter were so few that their platelet origin was uncertain.

Von Willebrand's Disease

Five cases of Von Willebrand's disease were examined, in which no abnormality of VM was detected (Table 1), although the interval between the onset of VM and fibrin

formation was slightly longer than in the normal series.

All had prolonged bleeding times, normal platelet counts and strong family histories of dominant non sex-linked inheritance. All, however, showed mild defects of anti-haemophilic globulin in their plasma.

Rosenthal's Syndrome (Plasma Thromboplastin Antecedent Deficiency). (Rosenthal, Dreskin & Rosenthal, 1955).

Three cases were examined with a coagulation defect of the same type as that described by Rosenthal et al. In all three, VM was different from that seen in all the other groups in that the onset of platelet clumping was delayed until just prior to fibrin formation, when the normal morphological changes suddenly took place. The interval between the start of VM and fibrin formation was shorter than in the normal series (Table 1).

CHAPTER IV

Factors Influencing Viscous Metamorphosis in Normal Native Plasma

The Effect of Various Anticoagulants on Normal VM

Normal platelet rich native plasma was treated with various additions to establish their influence on VM (Experiment 2; Appendix B. p.1) (Table II).

Heparin did not inhibit VM unless used in large amounts but did prevent fibrin formation. Neodymnium (3-sulpho-iso nicotinate) inhibited VM without preventing fibrin formation. Decalcifying salts inhibit both VM and fibrin formation, while soya bean trypsin inhibitor had no effect on VM but delayed fibrin formation.

Calcium and Viscous Metamorphosis

As sodium citrate, sodium oxalate and disodium versenate, used in strengths which prevent fibrin formation, also inhibited VM, it was necessary to determine whether the action of these salts was due to their calcium binding capacity or due to a direct action on the platelets themselves (Experiment 3; Appendix B, p.2) (Table III).

The results obtained do show that the inhibitory action of these salts is due solely to their ability to combine with calcium salts and that calcium is essential for normal VM.

TABLE II Experiment 2 Appendix B, p.1

The effect of various anticoagulants on viscous metamorphosis in normal platelet rich native plasma.

To 0.5 ml amounts of normal platelet rich native plasma were added 0.5 ml of additions to defined final concentrates.

Native Plasma	Nature of Addition	Final Conc. of Addition	Presence or Absence of Viscous Metamorphosis	Presence or Absence of Fibrin Clot	Clotting Time in mins.
Normal	Saline	-	+	+	4'45"
"	Heparin	0.1 u/ml	+	+ delayed	30'
"	"	1 u/ml	+	-	
"	"	5 u/ml	+	-	
"	"	20 u/ml	-	-	
"	Thrombodym (Neodymnium 3-sulpho iso-nico-tinate)	0.15%	-	-	
		0.075%	-	+ delayed	20'
		0.036%	+	+ delayed	11'25"
"	Sodium Citrate 3.8%	1 part to 9 parts plasma	-	-	
"	Sodium Oxalate 1.34%	1 part to 9 parts plasma	-	-	
"	Disodium Versenate 4.5%	1 part to 33 parts plasma	-	-	
"	Soya Bean Trypsin Inhibitor	1 mgm/ml	+	+ delayed	30'
		0.5 mgm/ml	+	+ delayed	"

TABLE III Experiment 3 Appendix B, p. 2

The role of calcium in viscous metamorphosis.

To 0.5 ml. platelet rich plasma previously treated with citrate, oxalate, or disodium versene was added 0.5 ml. M/40 $CaCl_2$ and the onset of VM and fibrin formation recorded.

Platelet Rich Plasma	Start of VM (in secs)	Fibrin Formation (in secs)	Interval (in secs)
Citrate 3.8%	60	226	166
Oxalate 1.34%	47	242	195
Disodium Versene 45%	45	301	256

Optimum Calcium Strength for Viscous Metamorphosis

The optimum strength of calcium required for VM and fibrin formation was determined (Experiment 4; Appendix B, p2) (Table IV). This experiment demonstrated that the addition of trace amounts of calcium, insufficient for normal coagulation, would allow VM to evolve normally.

Viscous Metamorphosis in Recalcified Citrate Plasma

It might reasonably be argued that the platelet changes observed in native plasma might be due to blood coagulation proceeding slowly during the separation of the 'native' plasma from whole blood. Theoretically this might occur as no anticoagulant is added to the blood. Therefore, parallel samples from the same venepuncture were examined; one was citrated immediately, the other was cooled rapidly and platelet rich native plasma obtained.

The citrated sample was centrifuged at 1400g for 10 minutes and platelet rich citrated plasma obtained. This was now recalcified and the platelet changes observed in relation to fibrin formation in parallel with the changes in the native plasma sample (Table V). (EXPERIMENT 5, Appendix B, p. 3).

It was at once evident that there was no significant difference between the two samples, and therefore it was unlikely that any latent coagulation was taking place while the native plasma was being prepared.

The Morphology of VM in Recalcified Citrate Plasma

When platelet rich citrated plasma was allowed to clot

TABLE IV Experiment 4 Appendix B, p. 2

The optimum strength of calcium for normal viscous metamorphosis.

To 0.5 ml. citrated platelet rich plasma was added 0.5 ml $CaCl_2$ in doubling dilutions. The development of normal VM and fibrin formation was observed.

Concentration of Added Calcium Chloride	Start of VM (in secs)	Fibrin Formation at 30 minutes
M/40 0.025M	30	+
M/80 0.012M	25	+
M/160 0.006M	30	+
M/320 0.003M	35	+
M/640 0.0015M	41	-
M/1280 0.00075M	42	-
M/2560 0.00037M	49	-
M/5120 0.00018M	58	-

TABLE V Experiment 5 Appendix B, p. 3

Comparison of the sequence of viscous metamorphosis in normal platelet rich native plasma and in normal platelet rich recalcified plasma.

	Platelet Rich Native Plasma + equal volume 0.85% saline		Platelet rich citrated plasma + equal volume of M/40 $CaCl_2$	
Sample	Start of VM (in secs)	Fibrin Formation (in secs)	Start of VM (in secs)	Fibrin Formation (in secs)
1	35	285	40	302
2	25	272	20	185
3	30	315	22	182
4	15	155	18	175
5	10	277	21	292

at 37°C after recalcification, VM developed with the identical morphological changes described in Chapter II (Figs. 14 & 15). But, after fibrin formation, the 'balloons' that appeared around the periphery of the platelet clumps were dark grey in colour (Figs. 16 & 17) and no clear white 'balloons' were observed.

Complement and Platelet VM

The inter-relationship of Complement with the blood coagulation system has invited the interest of numerous workers and, while it was originally thought that prothrombin and the C'_1 fraction of Complement were identical (Bordet 1920; Fuchs 1921), this has since been disproved (Boulanger & Rice 1951; Marx, Bayerle & Skibba 1949). It has been shown that the complement levels in serum, derived from cases with severe defects of antihaemophilic globulin, Christmas factor, Factor V and VII were similar to those found in normal serum (Sharp 1957).

In the experiments previously described, a process of cellular agglutination and partial lysis involving platelets has been observed and it was important to determine whether Complement was destroyed or fixed during this process. Therefore sera obtained from platelet rich and platelet free samples of normal plasma were examined for their titre of Complement.

As it was possible that Complement might act in this phenomenon along with a coagulation factor, similar preparations

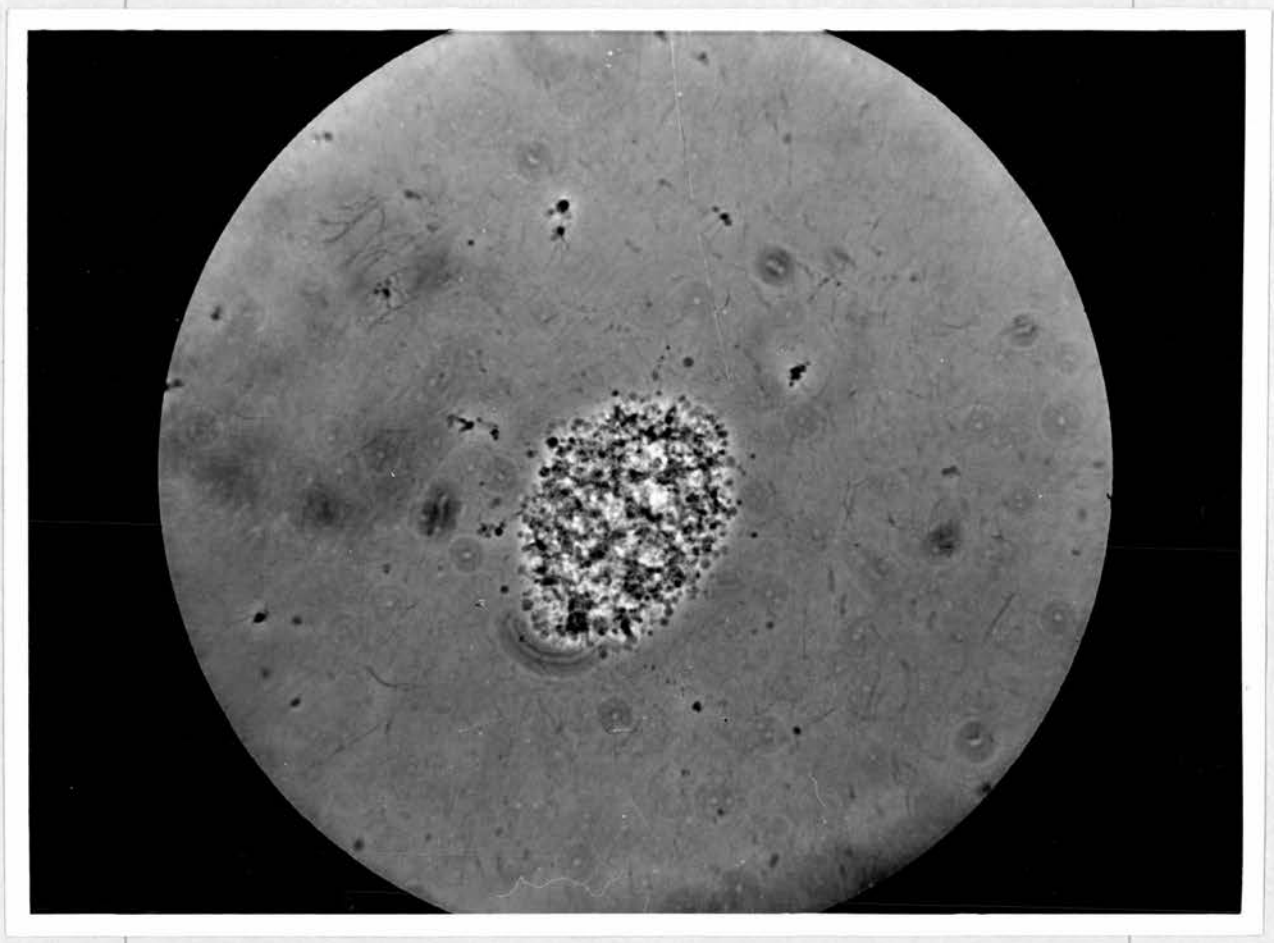

Figure 14
(Phase contrast 1100)

Early Viscous Metamorphosis

Early viscous metamorphosis (recalcified platelet-rich citrate plasma).

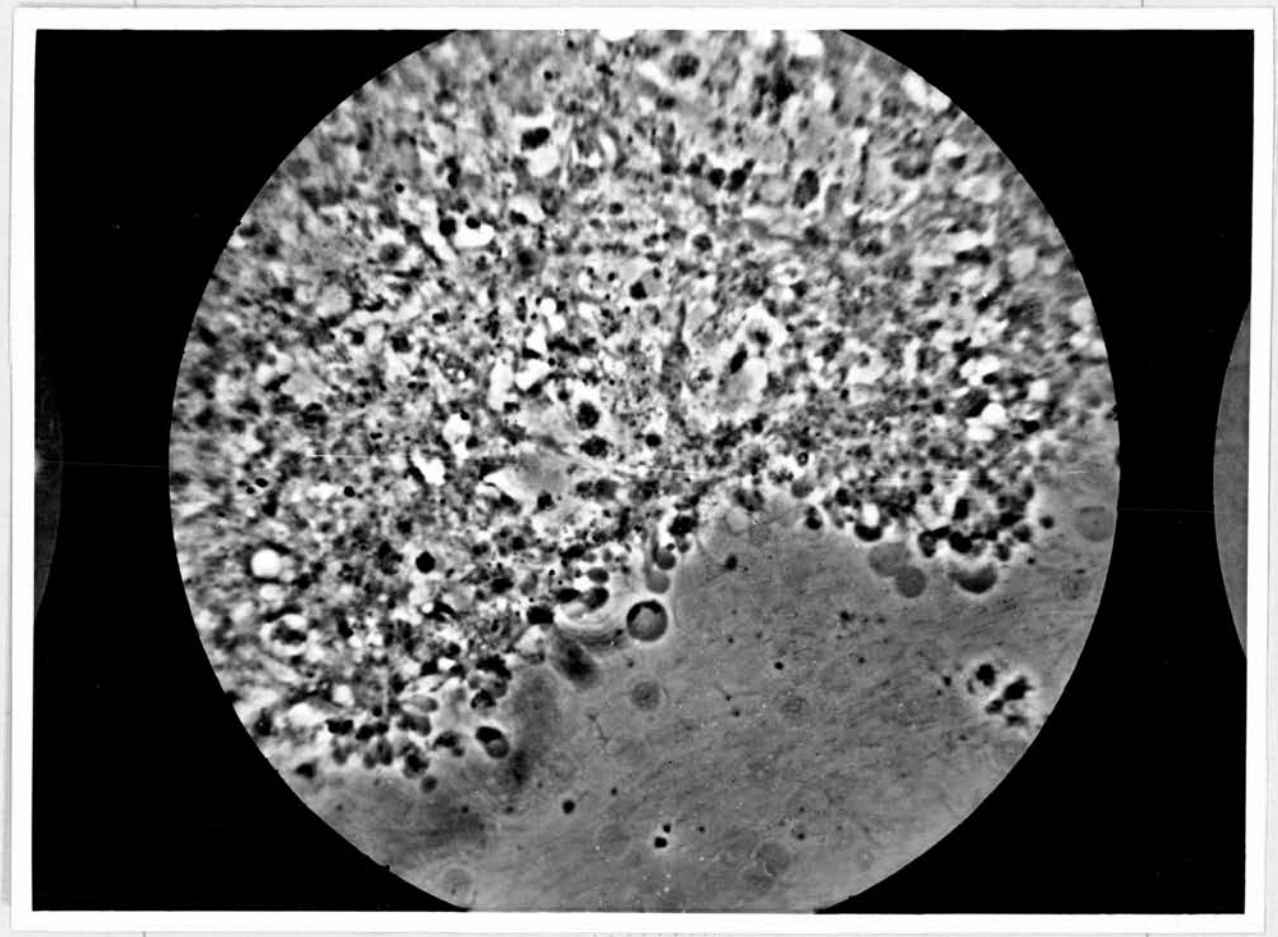

Figure 15
(Phase contrast x 5500)

Viscous Metamorphosis

Platelet fusion and lysis in typical viscous metamorphosis (recalcified platelet-rich citrate plasma).

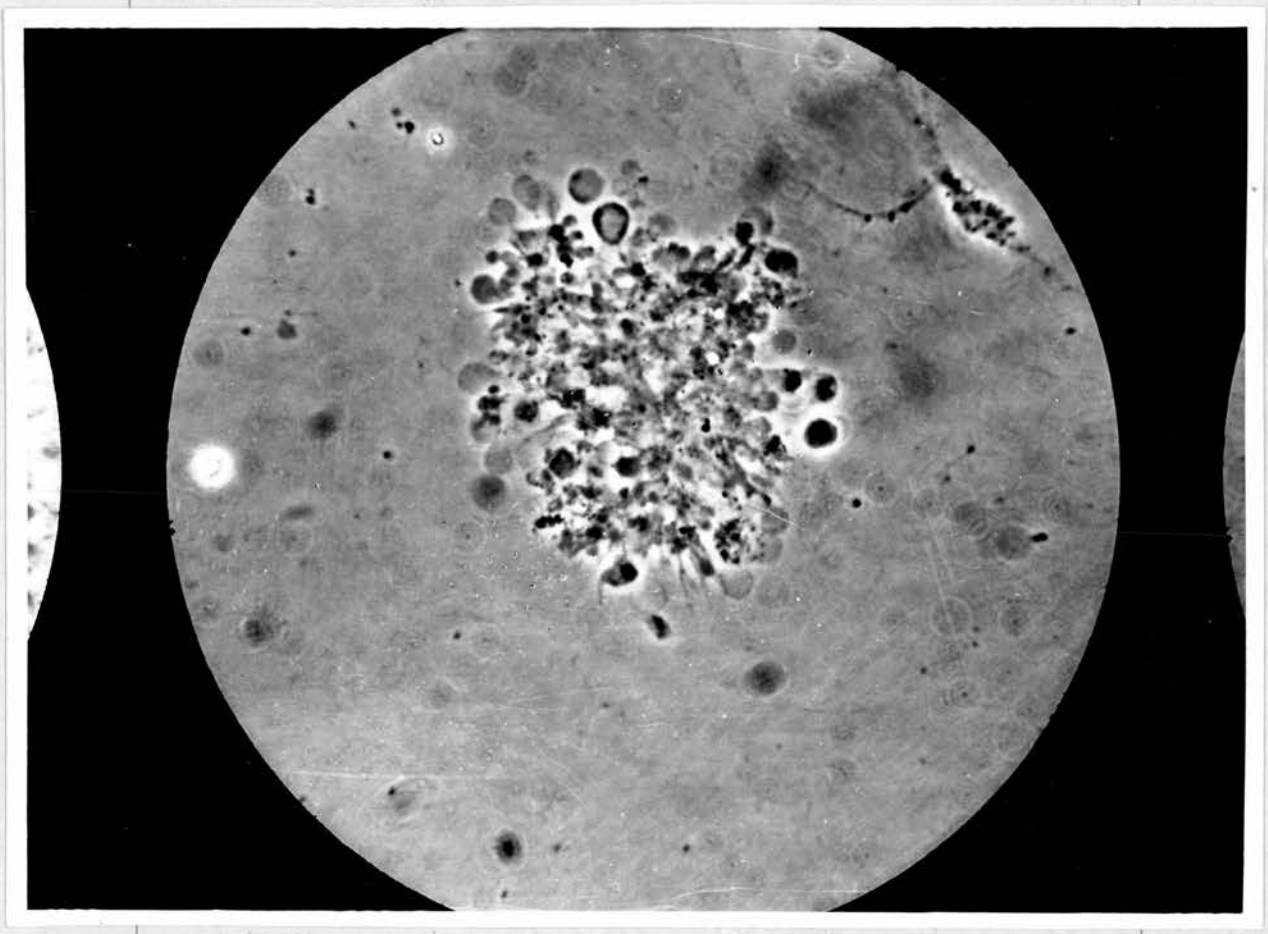

Figure 16
(Phase Contrast x 5500)

Figures 16 & 17

Late Viscous Metamorphosis

Dark grey 'balloons' forming around the periphery of the clump. The platelet granules have become arranged around the periphery of the 'balloons' (recalcified platelet rich citrate plasma).

Compare with Figs. 8, 9, 10).

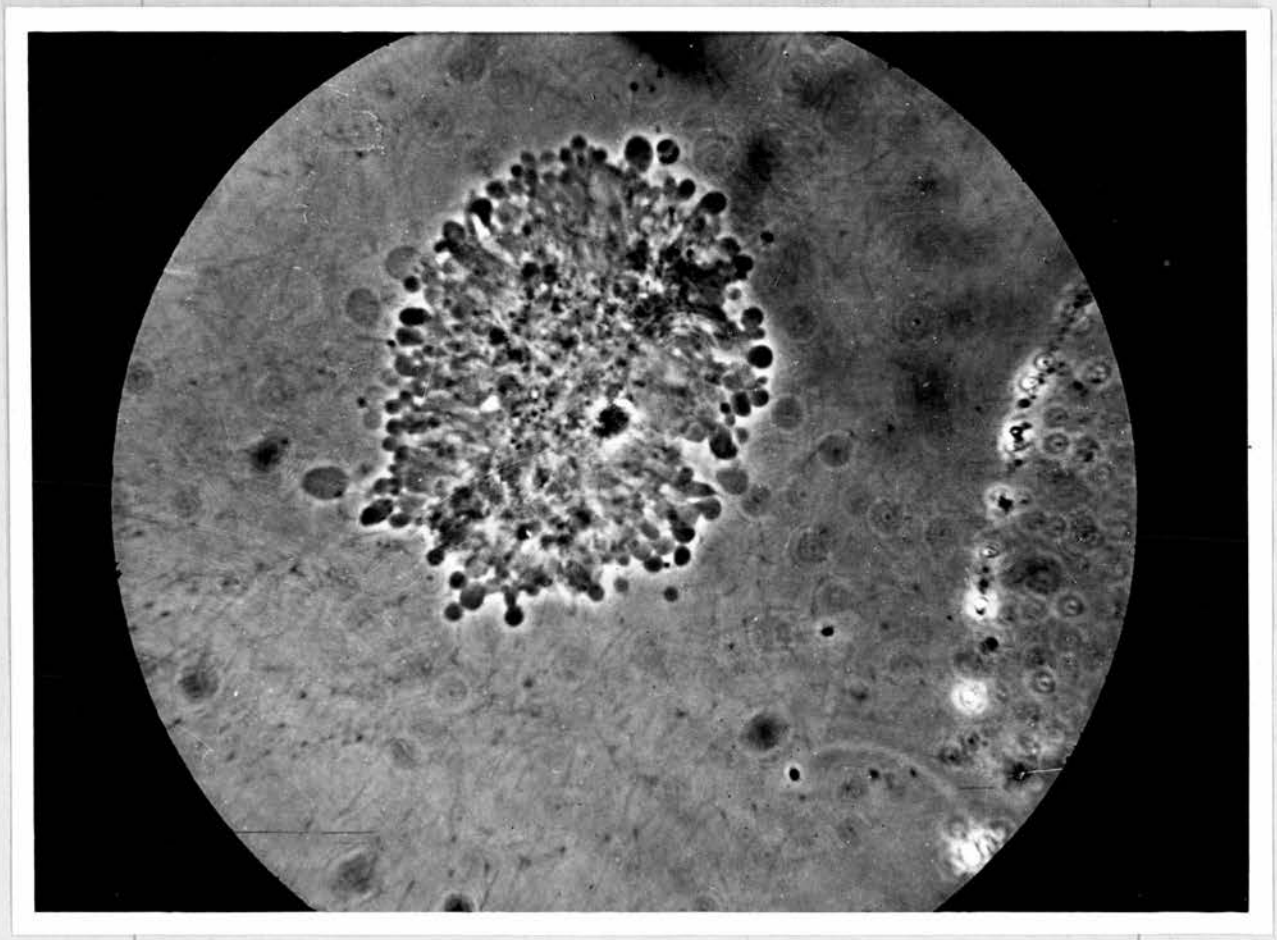

Figure 17

(Phase Contrast x 5500)

TABLE VI Experiment 6 Appendix B, p. 3.

The relationship of complement to platelet viscous metamorphosis

	1 Volume Serum + 2 Vols. 1.5% Sensitised Sheep Cells. End Point 50% Haemolysis														
	1/2	1/3	1/4	1/6	1/8	1/12	1/16	1/24	1/32	1/48	1/64	1/96	1/128	1/192	1/256
Normal Serum from Platelet Rich Plasma	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Normal Serum from Platelet Free Plasma	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Haemophilia Serum from Platelet Rich Plasma	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Haemophilia Serum from Platelet Free Plasma	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Christmas Serum from Platelet Rich Plasma	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Christmas Serum from Platelet Free Plasma	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Dindevan Serum from Platelet Rich Plasma	1.+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	2.+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Dindevan Serum from Platelet Free Plasma	1.+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	2.+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Factor V Serum from Platelet Rich Plasma	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Factor V Serum from Platelet Free Plasma	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-

were made from blood obtained from severe haemophilics, Christmas disease, congenital factor V deficiency and cases treated with Bindevan (factor VII deficiency). (Experiment 6; Appendix B p.3). Table VI).

The titre of Complement in serum derived from platelet rich plasma did not differ significantly from that obtained from platelet free plasma. This applied to both normal and abnormal plasma. Therefore it is not unreasonable to presume from these results that Complement is not essential for physiological platelet VM.

The results of the experiments described in Chapters III and IV may be summarised as follows:

(1) VM in both normal platelet rich 'native' plasma and recalcified plasma shows a definite time relationship to fibrin formation and the absence of certain essential coagulation factors prolongs the inverval between the onset of VM and the formation of fibrin.

(2) VM does not occur normally in thrombocytopenia or in plasma derived from cases of Rosenthal's syndrome. In haemophilia and in the presence of a circulating anticoagulant and heparin an apparent qualitative difference exists in that the platelet clumps once formed appeared to break up before fibrin appeared.

(3) Calcium is essential for VM and the optimal concentration required appears to be lower than that necessary for fibrin formation.

(4) VM is not inhibited by small concentrations of heparin but is by strong concentrations. Soya bean trypsin inhibitor similarly has no inhibitory effect but Neodymium will inhibit VM yet only delay fibrin formation.

(5) The morphological changes of VM developing in recalcified platelet rich citrate plasma, are essentially the same as those in normal native plasma.

(6) Complement does not appear to be utilised during the sequence of VM in plasma.

CHAPTER V

The Action of Serum on Platelets

When washed platelets are added to fresh serum they rapidly undergo changes identical to those recorded in Chapter II. This ability of serum to initiate viscous metamorphosis (VM) in washed platelets was originally described by Wright & Minot (1917) who, in a detailed and thorough investigation defined some of the properties of this serum factor and showed that, in the light of their knowledge of blood coagulation at that time, it was apparently a separate factor. The importance of their findings has been neglected until recently, when a revived interest in VM stimulated Bergsagel (1956) to repeat some of their experiments. He suggested that the factor in serum responsible for VM had similar properties to an intermediate product of thromboplastin formation and was probably the same. Stefanini & Silverberg (1951), however, considered that when platelets are added to fresh serum they stimulate the residual prothrombin to form thrombin which in turn will produce platelet agglutination (or VM). The action of thrombin on platelets will be discussed later but both Wright et al (1917) and Bergsagel specifically denied that thrombin was the factor concerned in serum VM.

This activity of serum was therefore re-examined in an attempt to decide which factor or factors, remaining in the serum after blood coagulation has occurred, were capable of

inducing VM in platelets. If whole blood was allowed to clot in glass at 37°C the ability of the resultant serum to produce VM was extremely variable.

The first step was to decide upon a technique which would allow reproducible results in terms of the speed and intensity of reaction.

Quite empirically it was decided to test sera derived from unaltered platelet rich native plasma clotted in glass tubes at 37°C and incubated for 1 hour after the formation of fibrin. The fibrin clot was removed and the resultant serum tested for activity against washed platelets (Appendix A, p.14). By using this technique it was found that normal sera were able to induce platelet agglutination and VM in suspensions of washed platelets within five minutes.

The Effect of Platelet Numbers on the Activity of Serum VM Factor

It has been claimed both by Wright & Minot (1917) that serum derived from platelet rich plasma had more agglutinating factor than that derived from platelet poor plasma. Luscher (1956) has claimed that the opposite is true.

In order to determine which of these claims was correct, the ability of sera from platelet rich and platelet free samples of the same plasma were tested and quantitatively assessed by titration (Experiment 17. Appendix B, p. 7).

This experiment has shown that the presence or absence of platelets in the plasma from which the serum is derived,

does not appear to influence the ability of serum to induce VM in the platelets (Table VII).

Washed Platelets and Serum VM

In assessing the ability of serum to agglutinate washed platelets, it was essential to determine whether repeated washing of the platelets could destroy their ability to react with the VM factor in the serum (Experiment 8, Appendix B p. 5). Washed platelet suspensions were prepared as described in Appendix A, p. 13. After each wash they were resuspended and a sample tested against fresh serum (Table VIII).

The platelets lost their ability to react with fresh serum after surprisingly few washes, and, in order to measure the ability of a serum to induce VM in platelets, the latter must not be washed more than twice. The process of washing must remove some substance from the platelets which must react with the serum factor. As the platelets appeared to be relatively unaltered morphologically, even after 10 washes, it is probable that this factor or factors are removed from the surface of the platelet.

The Influence of the Density of Platelet Suspensions on Serum Induced VM

Early in these experiments it was observed that VM could not be produced in weak platelet suspensions by fresh serum. Therefore an optimum density of platelets must exist before VM can evolve normally.

TABLE VII Experiment 7 Appendix B, p4.

The effect of platelet numbers on the activity of serum viscous metamorphosing factor.

Platelet rich and platelet free samples of normal native plasma were allowed to clot at 37°C and after incubation for 1 hour at 37°C the fibrin was removed and the serum was tested for VM factor activity.

Normal Plasma	Titre of VM Factor	
	Serum from Platelet Rich Plasma + Washed Normal Platelets	Serum from Platelet Free Plasma + Washed Normal Platelets
1	1/12	1/16
2	1/4	1/4
3	1/6	1/8
4	1/2	1/2
5	1/16	1/12

TABLE VIII Experiment 8 Appendix B, p.5.

The effect of washing on the ability of platelets to react with serum VM factor.

To 0.2 ml active normal serum was added 0.2 ml washed test platelet suspension and the presence or absence of viscous metamorphosis recorded

Number of Washings	Presence or Absence of Viscous Metamorphosis							
	Platelet Suspension							
	1	2	3	4	5	6	7	8
1	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+
3	+	+	-	+	+	+	+	+
4	-	+	-	+	+	+	+	+
5	-	±	-	+	±	-	+	+
6	-	-	-	-	±	-	+	±
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-

+ = VM evolving within 1 minute

± = VM evolving within 5 minutes

- = No VM after 10 minutes

By varying the density of platelet suspensions by doubling dilution, it was possible to determine approximately the number of platelets per cu.mm. that must be present before the phenomenon of agglutination can take place. Again fresh normal serum was used to induce platelet agglutination (Experiment 9, Appendix B p. 5).

The results obtained show that at least 60,000 platelets/cu.mm. must be present before normal VM can take place (Table IX).

These observations agree with those made when unaltered native plasma, prepared from thrombocytopenic blood, was allowed to clot.

The Morphology of VM Induced by Fresh Serum

The rate and sequence of VM induced by fresh normal sera in washed platelets was identical to that seen in normal clotting platelet rich plasma. Unlike that induced in platelet rich native or recalcified citrate plasma, or by thrombin, no fibrin formed in relation to the platelet clumps. The 'balloon' forms developed 20 - 30 minutes after the start of the reaction and they were dark grey in colour as illustrated in Figs. 16 and 17.

The VM Activity of Serum derived from Blood with Specific, Acquired, or Congenital Defects of Known Coagulation Factors

Bergsagel (1956) has suggested that the factor in serum responsible for inducing VM in washed platelets was the same as his intermediate product of thromboplastin formation. This

TABLE IX Experiment 9 Appendix B, p.5.

The minimum platelet density for normal viscous metamorphosis.

To 0.2 ml each concentration of platelets was added 0.2 ml fresh normal serum and the mixture incubated at 37°C for 10 minutes and the presence or absence of normal VM observed.

Final Density of Platelets/cu.mm.	Presence or Absence of Normal VM
248,000	+
185,000	+
126,000	+
71,000	+
58,000	±
39,000	-
24,000	-

latter factor was, he thought, produced by the interaction of antihaemophilic globulin, Christmas factor and calcium. If his theory were correct serum derived from blood deficient in antihaemophilic globulin or Christmas factor should contain little or no intermediate product and be unable to induce normal VM in normal washed platelets. This assumption would also be true if any other coagulation factor was responsible for serum VM.

Therefore serum derived from the blood of patients with specific congenital or acquired coagulation defects was examined for its ability to produce VM in suspensions of washed normal platelets (Experiment 10, Appendix B p.6).

Serum was prepared from cases of haemophilia, Christmas disease, factor V deficiency, induced factor VII deficiency, thrombocytopenia, Von Willebrand's disease and Rosenthal's syndrome (P.T.A. deficiency). In parallel the ability of platelets derived from these cases to react with normal serum was determined to see if platelets themselves might be insensitive to serum VM (Experiment 10).

The factor in serum responsible for VM was present in serum derived from blood deficient in antihaemophilic globulin, Christmas factor, factors V or VII or P.T.A., and all these samples appeared to show normal activity (Table X). The platelets derived from these patients with these specific defects reacted normally to fresh normal serum.

(a) The presence of VM activity in sera derived from blood with known congenital or acquired coagulation defects.

(b) The ability of platelet suspensions from duplicate samples of these abnormal bloods to undergo normal viscous metamorphosis

(a)		(b)	
Washed Normal Platelet Suspension + Test Serum		Normal Active Serum + Washed Test Platelet Suspension	
Source of Test Sera (Number Tested)	Presence or Absence of VM	Source of Test Platelets (Number Tested)	Presence or Absence of VM
Haemophilia (5)	+	Haemophilia (3)	+
Christmas Disease (4)	+	Christmas Disease (2)	+
Factor V Deficiency (1)	+	Factor V Deficiency (1)	+
Acquired Factor VII Deficiency (Dindevan) (3)	+	Factor VII Deficiency (1)	+
Thrombocytopenia (2)	+	Thrombocytopenia (2)	0
Von Willebrand's Disease (5)	+	Von Willebrand's Disease (2)	+
Rosenthal's Syndrome (1)	+	Rosenthal's Syndrome (1)	+

0 – It was impossible to make suitable platelet suspensions from these cases.

Thus serum VM factor did not seem to be dependent on the presence of any of these factors in the blood from which it had been derived nor did these results appear to confirm Bergsagel's hypothesis. The sera tested, however, were derived from clotted blood in which the various factors required for fibrin formation must have been generated, even if slowly. Under the experimental conditions, used to prepare the sera for testing, it is possible that Bergsagel's intermediate product might have been generated slowly, even in the presence of severe plasma factor defects and, by the time the sera were tested, be present in sufficient quantity to induce VM in washed platelets.

Thus, while the present experimental results do not appear to support Bergsagel's views, it is impossible to refute his hypothesis on this evidence alone.

Serum VM Factor and Thrombin

Wright & Minot (1917) and Bergsagel (1956) concluded that the platelet agglutinating factor in serum was not due to thrombin. However, Stefanini & Silverberg (1951) thought that platelets, when added to serum, were capable of generating thrombin from the residual prothrombin in the serum, which, in turn, could produce agglutination of the platelets.

Thrombin is defined as that fraction of blood, which, when added to fibrinogen will convert it to fibrin (Biggs & Macfarlane 1953). Therefore, if thrombin is present in fresh serum or is generated in serum by the addition of washed

platelets, it should be capable of converting fibrinogen to fibrin (Experiment 11/ Appendix B p.7) This experiment did not confirm the belief that the VM activity of fresh serum was due to the presence of residual thrombin, nor was it possible to show that any thrombin was generated in fresh serum when washed platelets, either alone or together with calcium, were added. (Table XI).

Complement and Serum VM of Platelets

It has been determined in Chapter IV that physiological VM does not require or fix Complement. As it was by no means certain that VM produced in washed platelets by serum was the result of the same mechanism as that observed in platelet rich native plasma, it was important to discover whether or not Complement was utilised during serum VM.

The titre of natural Complement in normal serum was compared before and after it has been used to induce VM in washed platelets. (Experiment 12. Appendix B p7). No significant reduction in the titre of Complement in serum was detected after it had induced VM. Therefore, it can be assumed that complement is not utilised during serum VM of platelets. (Table XII).

Properties of the VM Factor in Serum

In order to determine the physical properties of the platelet agglutinating factor in serum, fresh serum of proven potency was re-tested after exposure to heat, absorption with inorganic salts, after the addition of certain anticoagulants

TABLE XI Experiment 11 Appendix B, p.7.

The relationship of serum VM factor to thrombin.

Test mixtures were added to a solution of pure fibrinogen and incubated together at 37°C. The presence or absence of fibrin formation was recorded at varying intervals.

Test Mixture	Presence or Absence of Fibrin				
	Incubation Time in Mins.				
	5	30	60	120	240
Fibrinogen 0.2 ml + Active Serum 0.2 ml + Saline 0.2 ml	-	-	-	-	-
Fibrinogen 0.2 ml + Human Thrombin 0.2 ml + Saline 0.2 ml	+				
Fibrinogen 0.2 ml + Active Serum 0.2 ml + Washed Normal Platelets 0.2 ml.	-	-	-	-	-
Fibrinogen 0.2 ml + Active Serum 0.2 ml + Washed Normal Platelets 0.2 ml. + M/40 $CaCl_2$ 0.2 ml.	-	-	-	-	-
Fibrinogen 0.2 ml + Active Serum 0.2 ml + M/40 $CaCl_2$ 0.2 ml	-	-	-	-	-

TABLE XII Experiment 12 Appendix B, p. 7

The relationship of complement to serum induced viscous metamorphosis.

Complement Titres 1 Vol. serum + 2 vols. 1.5% sensitised sheep cells.
End Point 50% Haemolysis.

Serum B / A	1/2	1/3	1/4	1/6	1/8	1/12	1/16	1/24	1/32	1/48	1/64	1/96	1/128	1/192	1/256
Normal B	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
A	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Normal B	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
A	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Normal B	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
A	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Normal B	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
A	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Normal B	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
A	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−

B = Before inducing VM

A = After inducing VM in washed platelets.

and after differential absorption with platelets and red cells (Experiment 13. Appendix B, p8).

Serum VM factor was found to have certain well defined properties (Table XIII). It is unstable on storage at room temperature, all the activity having disappeared within 24 hours. It is only partially destroyed by heating to 56°C for 30 minutes, being still capable of inducing agglutination but not VM. It was completely destroyed by treating at the same temperature for 1 hour. This agrees with the findings of Wright & Minot (1917); Stefanini & Silverberg (1951). The factor is adsorbed by barium sulphate and aluminium hydroxide gel and is inactivated by sodium citrate, oxalate and disodium versene. Calcium is obviously essential for the action of this factor, as the ability of the serum to produce VM, when destroyed by citrate or oxalate, is restored by the addition of calcium. Heparin destroyed the action of this factor while thrombodym and soya bean trypsin inhibitor did not. These actions of heparin and thrombodym were the exact opposite of those obtained when these anticoagulants are added to platelet rich native plasma. (See Table II and p. 44). This factor is apparently consumed or adsorbed during VM and can be removed by dialysis against normal saline for 18 hours.

Isolation of the Serum Factor responsible for VM

In order to isolate the factor responsible for VM, globulin, ammonium sulphate, and phosphate fractions of active fresh serum were made and the resultant fractions were assayed

TABLE XIII Experiment 13 Appendix B, p.8.

The physical properties of serum VM factor.

0.2 ml active serum before and after various treatment was added to 0.2 ml washed normal platelet suspension together with 0.1 ml addition. The presence or absence of normal VM was recorded after 5 mins. incubation at 37°C.

Serum	Addition (Concentration)	Presence or Absence of VM
Normal	Saline	++
"	Sodium Citrate 1.9%	-
"	Sodium Oxalate 0.67%	-
"	Disodium Versene 0.8%	-
"	Heparin 5 u/ml	-
"	Thrombodym 2.5%	+
"	Soya Bean Trypsin Inhibitor 5 mg/ml	+
Normal Heat Treated 56°C. 30'	Saline	± (Agglutination)
" Heat Treated 56°C. 60'	"	-
" $Al(OH)_3$ absorbed	"	-
" $BaSO_4$ absorbed	"	-
" Absorbed Xeo carb. 10 mins.	"	± (Agglutination)
" Absorbed Normal Platelets	"	-
" Absorbed Red Cells	"	+
" Dialysed 18 hrs. at 4°C	"	-
" " "	M/20 $CaCl_2$	-
" Stored 24 hrs. RT	Saline	-
" " " " -12°C	"	+
" " 7 days "	"	-
" +1/10 3.8" sodium citrate	7/20 $CaCl_2$	++
" +1/10 1.34% sodium oxalate	"	++

for their ability to produce VM in washed platelets. (Experiment 14. Appendix B. p.10). The results are recorded in Table XIV.

The Euglobulin fraction showed an increased activity towards normal platelets. The retention of activity in this fraction proves that the active factor is either a protein or closely associated with protein.

Both the ammonium sulphate and phosphate fractions from active serum showed no demonstrable activity against platelets. As these inactive fractions were all dialysed against citrate-saline for 24 hours it was possible that all or part of the active fraction was dialysable. It has been shown (p. 56) that active serum dialysed against saline overnight also lost its activity. It is well recognised that calcium can be dialysed from serum and, as calcium is essential for serum induced VM, calcium was added to the various protein fractions obtained. In all instances it failed to restore activity against platelets.

These observations suggest that the active fraction in serum is made up of a protein associated with a dialysable fraction that does not appear to be calcium. The serum factor responsible for VM was very unstable on storage unless the serum was frozen at -12°C (Table XIII). Therefore it cannot be excluded that the apparent loss of activity, during dialysis or salt fractionation or both, was not due to this instability rather than to the removal of a dialysable fraction.

The concentration of serum VM factor.

To 0.2 ml washed platelet suspension was added 0.2 ml protein fraction together with 0.2 ml addition. The presence or absence of VM was recorded after 5 mins. incubation at 37°C.

Serum Fraction	Addition	Presence or Absence of Viscous Metamorphosis
Whole serum	Saline	++
Euglobulin Fraction	Saline	++
Ammonium Sulphate Fraction		
0-25% Saturation	Saline	-
25-33% "	"	-
33-50% "	"	-
0-25% "	M/40 $CaCl_2$	-
25-33% "	"	-
33-50% "	"	-
Phosphate Fractions		
1.0 M	Saline	-
1.5 M	"	-
2.0 M	"	-
1.0 M	M/40 $CaCl_2$	-
1.5 M	"	-
2.0 M	"	-
Whole Serum after dialysis 18 hrs. at 4°C	Saline	-
"	M/40 $CaCl_2$	-

The experiments recorded in this chapter may be summarised as follows:

(1) The activity of serum VM factor is not influenced by the number of platelets in the blood from which it is derived.

(2) Before serum can induce VM, an optimum density of platelets must be present and a co-factor must exist on the surface of these platelets.

(3) This property of serum does not appear to be dependent on A.H.G., Christmas factor, factors VII or Rosenthal's factor (P.T.A.) in the blood from which it is derived. Nor can this action of serum be identified with thrombin. It cannot be excluded that an intermediate product of thromboplastin might be responsible for VM in all sera, both normal and abnormal.

(4) The serum factor is unstable on storage, destroyed by heat, inactivated by barium sulphate and aluminium hydroxide, inactivated by decalcifying salts and heparin but not by neodymium or soya bean trypsin inhibitor, consumed during VM, present in the globulin fraction but not in salt fractions of active serum.

These findings agreed in many respects with those of Wright & Minot (1917). They found that in their experiments, the serum from platelet-rich plasma was more active than that from platelet-free but, on the other hand, they found that the serum from thrombocytopenic blood was active - a finding confirmed in this Chapter. They also denied that this factor was consumed on the addition of platelets. This latter discrepancy may have been due to their failure to add a sufficient quantity of platelets to absorb all VM factors.

CHAPTER VI

Viscous Metamorphosis and Intermediate Products of Blood Coagulation (Including Thrombin)

Bergsagel's Intermediate Product of Thromboplastin Formation

As has already been discussed, Bergsagel, while trying to unravel the initial stages of blood coagulation, produced experimentally a factor capable of inducing VM in washed platelets (Bergsagel 1956). This he produced by incubating a mixture of pig A.H.G., aged serum and calcium, and he thought it to be an intermediate product of intrinsic thromboplastin formation.

If his hypothesis were correct, it would follow that VM should not take place normally in patients with haemophilia or Christmas disease.

As the experiments performed in the previous chapters did not confirm this it was thought to be worth while to repeat Bergsagel's experiments.

Antihaemophilic globulin derived from pig plasma (Bidwell 1955b), calcium and various aged sera were incubated together for 20 minutes and samples of the mixture then tested for their ability to produce VM in washed normal platelet suspensions (Experiment 15. Appendix B, p.13).

The results of this experiment suggested that Bergsagel's supposition was partially correct but it appeared that any

TABLE XV Experiment 15 Appendix B, p.13.

The composition of Bergsagel's VM factor.

Pig A.H.G. (1 mgm/ml) 0.1 ml + M/40 $CaCl_2$ 0.1 ml + addition 0.1 ml were incubated together for 20 minutes at 37°C and then 0.2 ml of the mixture added to 0.2 ml x2 washed platelets and the presence or absence of viscous metamorphosis recorded after incubation at 37°C for 5 mins.

Incubation Mixture	Nature of Addition	Presence or Absence of VM of Washed Platelets
Pig A.H.G.	Saline	-
Saline	1/5 aged normal serum	-
Saline	Saline	-
Pig A.H.G.	1/5 aged normal serum	+
"	1/5 aged Christmas serum	+
"	1/5 aged haemophilic serum	+
"	1/5 aged factor VII deficient serum	+
"	1/5 aged serum containing a circulating anti-coagulant	+
"	1/5 aged P.T.A. deficient serum	+
"	H.T. Normal serum 56°C for 1 hour	-
"	$BaSO_4$ absorbed normal serum	-

H.T. = Heat treated

aged sera could replace normal aged sera (Table XV). The absence of Christmas factor or factor VII from the serum component of the mixture did not influence the results. This was at variance with Bergsagel's findings (Bergsagel 1956). These experiments differed from Bergsagel's in one important feature, namely, the omission of buffer from the incubation mixtures. Bergsagel added 0.4 ml Imidazole buffer to his mixtures, thus producing a twofold dilution of all the components. This, it was felt, might produce false negative results simply by reason of dilution. The serum factor that was active in these mixtures was destroyed by heating to 56°C for 1 hour and by barium sulphate adsorption.

The Properties of Bergsagel's Factor

The properties of the VM factor generated in the above mixtures were determined (Experiment 16. Appendix B, p.13). The results are recorded in Table XVI.

When these properties are compared with those of serum VM factor (Table XIII) they are similar with one important exception; the VM factor in the present experiments did not require the presence of calcium for its activity.

During the incubation of these mixtures of A.H.G., serum and calcium, a fibrin clot formed before the twenty minute period had elapsed. This must have originated from the fibrinogen contaminating the pig A.H.G. concentrate (Bidwell 1955b) and, if this were so, it followed that thrombin must have been generated in the incubation mixture. Whether the latter was derived from residual prothrombin in the serum

The properties of Bergsagel's VM factor.

0.2 ml of the incubation mixture (I.M.) (Pig A.H.G., Serum, Calcium) was added to 0.2 ml x2 washed normal platelets together with 0.1 ml addition and the presence or absence of VM recorded after incubation at 37°C for 5 mins.

Incubation Mixture	Addition	Presence or Absence of VM
I.M.	Saline 0.85%	+
"	Sodium Citrate 1.9%	+
"	Sodium Oxalate o.67%	+
"	Disodium Versene 0.8%	-
"	Heparin 5 u/ml	-
"	Thrombodym 2.5%	+
"	Soya Bean Trypsin Inhibitor 5 mgm/ml	+
I.M. Heat Treated 56°C. 30'	Saline 0.85%	-
I.M. $Al(OH)_3$ absorbed	"	-
I.M. $BaSO_4$ absorbed	"	-
I.M. Absorbed normal platelets	"	-
I.M. Absorbed red cells	"	+
I.M. Stored 24 hrs. Room Temp.	"	-
I.M. Stored 24 hrs. -12°C	"	-

fraction or from prothrombin contaminating the animal A.H.G. was uncertain.

Bergsagel (1955a) also noted this phenomenon but, as he did not think that thrombin could induce VM in platelets, he ignored it.

Thrombin and Platelet VM

The effect of thrombin on platelets has been the subject of controversy for many years. The evidence of the main protagonists has been described in Chapter I.

The Action of Thrombin on Washed Platelets

Bergsagel's whole hypothesis on the ability of intermediate products of thromboplastin formation to induce VM in washed platelets depended on his assumption that thrombin could not produce similar changes. He considered that the results of some workers could be explained by their use of bovine thrombin, which he considered produced platelet clumping by virtue of a non-specific interspecies reaction or by its content of unsuspected intermediate products of blood coagulation.

Commercial thrombin is usually bovine in type and, in this country, commercial thrombin (Maw's Topical) is made by absorbing prothrombin from oxalate bovine plasma with barium sulphate. The prothrombin is eluted from the barium powder by washing with sodium citrate and the eluate is converted to thrombin by the addition of calcium and lung thromboplastin. After dialysis the activity is preserved by

lyophilisation. This process produces an extract which, having definite thrombin activity, is probably contamihated by other coagulation factors.

Human thrombin, prepared by the method of Biggs & Macfarlane (1953) by precipitation from plasma at pH 5.3, is relatively pure, although it cannot be excluded that it also contains certain unspecified contaminants.

Thrombin and Washed Human Platelets

Bergsagel's experiments were repeated using identical methods and his findings were confirmed (Experiment 17. Appendix B/ p.14) but morphologically the changes induced by bovine thrombin were those of simple platelet agglutination and the typical changes of VM did not take place (Table XVII).

The Effect of Human Thrombin on Platelet Rich Plasma

When human thrombin was added to normal platelet rich citrate plasma fibrin was formed rapidly. When strong concentrations (10-20 units/ml) were used, no platelet changes took place before fibrin formation occurred but minimal concentrations (0.5 - 2 units/ml), just adequate for the conversion of fibrinogen to fibrin, induced rapid platelet VM at an appreciable interval before fibrin appeared.

The ability of varying anticoagulants to modify this reaction was studied (Experiment 18. Appendix B, p.14). These results showed that this phenomenon was apparent in platelet rich oxalate and citrate plasma but not in platelet rich versene plasma. The addition of heparin, even in small

TABLE XVII Experiment 17 Appendix B, p.14.

The effect of thrombin on <u>washed</u> normal human platelets.

Thrombin 20 u/ml 0.2 ml was added to normal human washed platelets (x2) together with addition 0.2 ml, and the presence or absence of viscous metamorphosis observed after 5 mins. incubation at 37°C

Nature of Thrombin	Nature of Addition	Presence or Absence of VM
Human Thrombin (Biggs & Macfarlane 1953)	Saline	-
	M/40 $CaCl_2$	-
Bovine Thrombin (Maws Topical)	Saline	- (+ Agglutination)
	M/40 $CaCl_2$	- (+ Agglutination)

concentrations, completely inhibited this reaction, but Neodymnium (3-sulpho-iso nicotinate) and soya bean trypsin inhibitor did not (Table XVIII).

The pattern of results, obtained with the latter three anticoagulants, was similar to that seen when they were added to fresh serum (see Table XIII). Unlike serum VM the effect of thrombin on platelets was not neutralised by sodium citrate or sodium oxalate, and therefore calcium did not appear to be necessary for this reaction. These properties were similar to those found when Bergsagel's Intermediate Product was tested. The inability of human thrombin to induce VM in platelet rich versene plasma was probably due to the 'heparinoid' or anti-thrombin action of this anticoagulant (Zucker 1954; Triantaphyllopoulos, Quick & Greenwalt 1955).

The Morphology of VM Induced by Thrombin

The platelet changes were similar to those described in Chapter II but, like those in recalcified platelet rich plasma, the 'balloon-like' swellings produced, after fibrin formed, were dark grey in colour. The latter change was fully developed five minutes after fibrin formed and the entire reaction took place more rapidly than that observed in platelet rich native or recalcified citrate plasma (Fig. 18).

Thrombin VM Co-Factor

As human thrombin had no effect on washed platelets separated from their plasma, but did induce VM in platelet rich plasma, it appeared reasonable to suppose that some co-factor

TABLE XVIII Experiment 18 Appendix B, p. 14

The effect of certain anticoagulants on thrombin induced VM

0.4 ml human thrombin (1 u/ml) was added to 0.5 ml platelet rich plasma together with 0.1 ml addition and the presence or absence of VM evolving before fibrin formation recorded.

Normal Platelet Rich Plasma	Addition Concentration	Presence or Absence of VM
Normal Citrate	Saline 0.85%	+
Normal Oxalate	Saline 0.85%	+
Normal Versene	Saline 0.85%	-
Normal Citrate	Heparin 10 u/ml	-
Normal Citrate	Neodymnium 2.5%	+
Normal Citrate	Soya Bean Trypsin Inhibitor 10 mgm/ml	+

TABLE XIX Experiment 19 Appendix B, p.15

The ability of thrombin to promote VM in abnormal platelet rich plasma.

To 0.5 ml platelet rich citrate plasma was added 0.4 ml thrombin (1 u/ml) and the presence or absence of VM evolving prior to fibrin formation was recorded.

Nature of Platelet Rich Citrate Plasma	Presence or Absence of VM prior to fibrin formation
Normal	+
Haemophilia	+
Christmas Disease	+
Factor V Deficiency	+
Dindevan treated (Factor VII deficiency)	+
Rosenthal's Syndrome (P.T.A. deficiency)	+

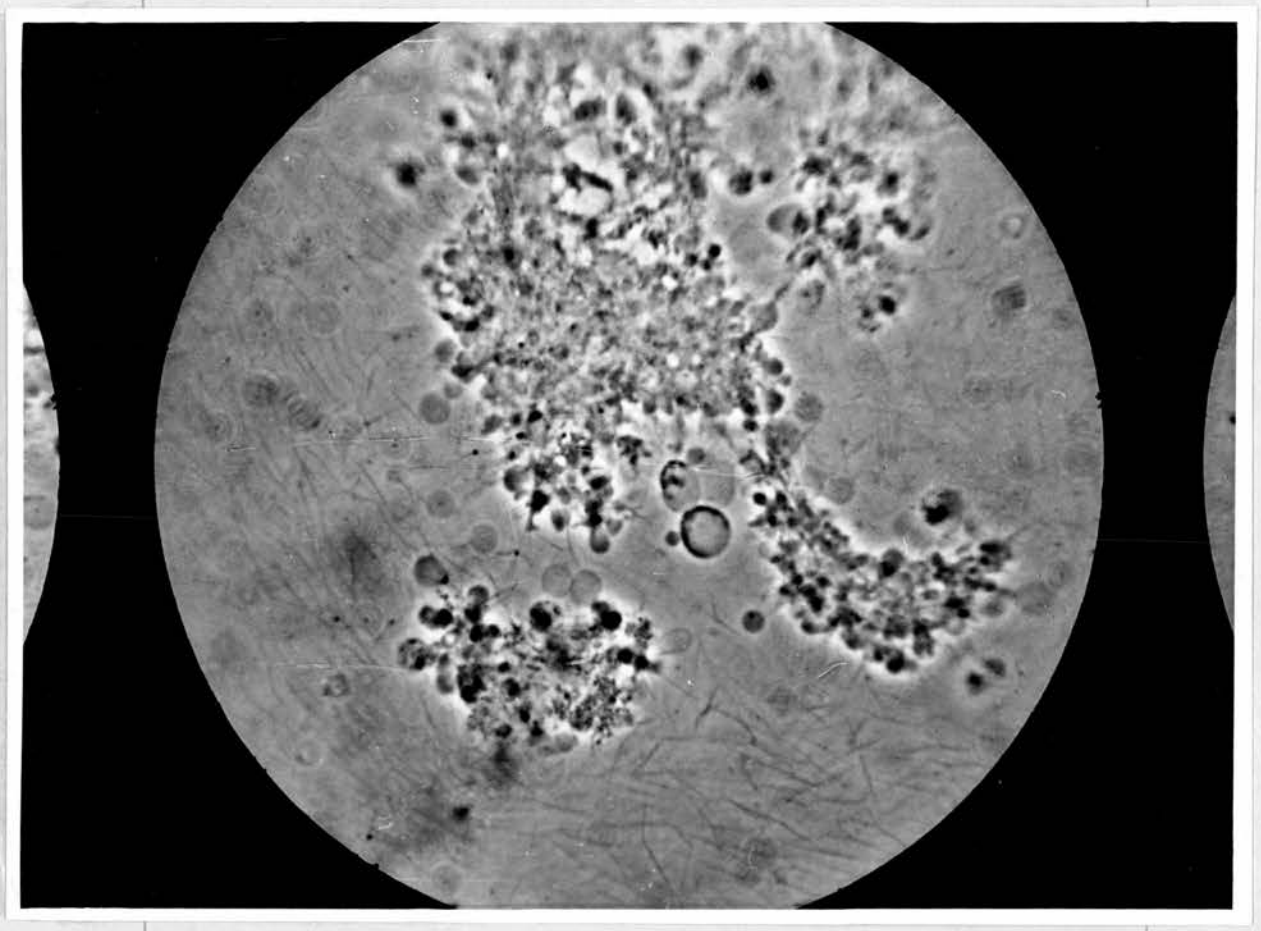

Figure 18
(Phase contrast x 5500)

Viscous Metamorphosis

Thrombin induced viscous metamorphosis in platelet-rich citrate plasma. The typical platelet fusion and lysis has occurred. The peripheral 'balloons' are dark grey in colour (compare Figs. 8, 9, 10 and 16).

must exist in the plasma which was essential for the action of thrombin on the platelets. This co-factor could have been one of the existing and recognised coagulation factors.

Human thrombin was therefore added to platelet rich citrated plasma prepared from cases with specific coagulation defects (Experiment 19. Appendix B, p.15). (Table XIX).

In every instance platelet VM was induced and therefore the thrombin VM co-factor could not be A.H.G., Christmas factor, factors V or VII or P.T.A.

It was still possible that prothrombin, fibrinogen, or some other as yet undertermined plasma or serum factor might have co-factor activity. Thrombin was therefore added to a series of mixtures of washed platelets and plasma, serum, barium sulphate adsorbed plasma and pure fibrinogen (Experiment 20. Appendix B, p.15). (Table XX).

These results showed that purified fibrinogen had co-factor activity but the plasma fibrinogen did not (Table XX). Thus either these two fibrinogens were different or purified fibrinogen contained some other factor not present in freshly prepared platelet free citrate plasma.

Animal A.H.G. and Thrombin Co-factor Activity

These preparations, made from pig and bovine blood, (Bidwell 1955 a & b), contain a large amount of fibrinogen and are prepared by a similar method to that used to separate purified fibrinogen from plasma.

TABLE XX Experiment 20 Appendix B, p.15.

The nature of thrombin VM co-factor.

0.1 ml human thrombin (7 u/ml) was added to 0.4 ml washed normal platelets + 0.1 ml addition, and the presence or absence of typical VM recorded.

Test System	Addition	Presence or Absence of VM
Human thrombin + washed normal platelets	Saline	-
"	High spun normal citrate plasma	-
"	$BaSO_4$ absorbed normal plasma	-
"	Aged serum	-
"	Pure Fibrinogen	+

Therefore these were tested for co-factor activity by a similar system to that used in the previous experiment.

They too were found to have thrombin VM co-factor activity. This result suggested that either salt fractionation confers co-factor activity on human or animal fibrinogen or that concentrated A.H.G. together with fibrinogen contained some other factor not present in high spun platelet-free plasma.

Platelet Factors and Thrombin Co-factor Activity

Both the purified fibrinogen and the animal A.H.G. concentrates were prepared from platelet rich citrated plasma; the human fibrinogen from stored blood bottle plasma and the animal preparations from platelet rich citrated pig or bovine blood.

Therefore thrombin co-factor activity might arise from the platelets or some breakdown products thereof. Extracts of human platelets were therefore prepared before and after washing and tested for their co-factor activity. (Experiment 21. Appendix B, p.16).

These results do suggest that thrombin co-factor activity is in fact related to the platelets in some way (Table XXI). Whatever the factor, it is removed instantly from the platelets by washing, and thus it is unlikely that it is one of the known platelet coagulation factors which are extractable from well-washed platelet suspensions (Ware et al 1948; Creveld & Paulssen 1951 & 1952; Deutsch et al 1955). If this view were correct, it was thought likely that thrombin co-factor

TABLE XXI Experiment 21 Appendix B, p. 16.

Thrombin VM co-factor activity in platelet extracts.

Platelets were separated from normal plasma and protein extracts made (1) immediately and (2) after washing three times in normal saline. 0.1 ml of these extracts was tested for VM co-factor activity, human thrombin 0.1 ml (7 u/ml) and (x2) washed normal human platelets (0.4 ml) being used as the test system.

Test System	Platelet Extracts	Presence or Absence of VM
Human thrombin + washed normal platelets	1	+
	2	-
Saline + washed normal platelets	1	-
	2	-

material could be released from platelets into plasma on storage. Such a mechanism has been suggested by Bounameaux (1955) who has found platelets lost their ability to react with thrombin on storage in saline.

Washed platelets were added together with thrombin to high spun glass-stored plasma (blood bank plasma) in order to determine whether this, unlike high spun fresh plasma, contained VM factor (Experiment 22. Appendix B, p.17).

This experiment confirmed that thrombin co-factor is a substance closely related to the platelets (Table XXII). Its exact nature has still to be determined and its properties must be more fully elucidated before it can be decided whether it is the same as that described by other workers (Luscher 1956; Bounameaux 1957 b).

It has been assumed during all these experiments that the human thrombin used was pure and that the morphological platelet changes were due to the direct action of thrombin. This assumption has been made by other workers (Luscher 1956; Bounameaux 1957b), but, while these thrombin extracts are thought to be pure, it cannot be excluded that those extracts used in the present experiments, and those of other workers, did not contain some other, as yet unidentified, coagulation factors which might be responsible for platelet VM rather than thrombin.

Summary

The experiments described in this chapter have shown that:

(1) Thrombin can induce VM in platelets and requires a

TABLE XXII Experiment 22 Appendix B, p. 17.

The relationship of thrombin VM co-factor activity to platelets.

0.1 ml high spun platelet-free fresh plasma and high-spun platelet-free stored plasma were in turn added to a mixture of human thrombin 0.1 ml (7 u/ml) and 0.4 ml washed normal human platelets. The presence or absence of VM evolving before fibrin formation was recorded.

Test System	Addition	Presence or Absence of VM
Human thrombin + washed normal human platelets	High spun fresh citrate plasma	-
Human thrombin + washed normal human platelets	High spun stored citrate plasma	+

co-factor for this action;

(2) this co-factor is probably derived from the blood platelets;

(3) artificial coagulation systems containing animal A.H.G., serum and calcium can generate a factor which will induce VM in washed platelets. These mixtures can generate thrombin and it is probable that this, and not any other intermediate product of blood coagulation, is responsible for inducing VM in platelets. The fact that animal A.H.G. has co-factor activity supports this contention.

It is convenient at this stage to compare the factors which inhibit or prevent VM in each of the three experimental systems so far described (Table XXIII).

The variations in these results will be discussed in Chapter VIII.

TABLE XXIII

A comparison of viscous metamorphosis in the three systems used experimentally to study this phenomenon.

Type of Case from which Platelet Suspension or Serum was Derived	Presence or Absence of Normal Viscous Metamorphosis		
	Platelet Rich Native Plasma	Serum + Washed Normal Platelets	Thrombin + Platelet Rich Citrated Plasma
Normal	+	+	+
Haemophilia	+	+	+
Christmas Disease	+	+	+
Congenital Factor V Deficiency	+	+	+
Induced Factor VII Deficiency	+	+	+
Thrombocytopenia	-	+	-
Rosenthal's Syndrome	-	+	+
The effect of added anticoagulants can be compared			
Sodium Citrate 3.8%	-	-	+
Sodium Oxalate 1.34%	-	-	+
Disodium Versene 4.5%	-	-	-
Heparin 1 u/ml	+	-	-
Thrombodym 0.15%	-	+	+
Soya Bean Trypsin Inhibitor 1 mgm/ml	+	+	+
Typical morphology	+	+	+
Co-factor required	0	+	+

CHAPTER VII

Platelet Agglutination or Viscous Metamorphosis By Other Factors Which Directly Influence Blood Coagulation

Several factors have been described which, when added to blood, will accelerate the speed of coagulation. The action of the majority has been explained satisfactorily but their influence on the platelets has not been defined. The evolution of techniques to determine whether they had any direct action on platelets was rendered difficult by the fact that platelets undergo rapid VM in normal unaccelerated blood coagulation. Therefore, in some instances, it has been possible only to determine whether these factors can agglutinate or produce VM of platelets suspended in plasma or separated from plasma as washed suspensions.

Glass Surface

Lister (1863) was the first to stress that contact with a foreign surface was an essential feature of blood coagulation. In all experiments to study in vitro blood coagulation, glass surface plays an important role and it is recognised that blood clotted in glass does so much more rapidly than when clotted in tubes coated with paraffin or silicone (Jaques, Fidlar, Felsted & MacDonald 1946). The role of platelets in this phenomenon has been the subject of controversy. It has been established that, if platelets are removed carefully and completely from plasma, this plasma will not clot even on

contact with glass (Jaques et al 1946; Paton, Ware & Seegers 1948).

Lozner, Taylor & MacDonald (1942) considered that the effect of foreign surfaces on blood coagulation was independent of the platelets and that an inactive precursor existed in blood which, on activation by glass contact, had thromboplastic activity. This view was supported by Jaques et al (1946) and Hartman, Conley & Lalley (1949), who noted that platelet free plasma took longer to clot in silicone than in glass and that increasing the glass surface shortened the clotting time of plasma regardless of the platelet concentration. Hartman et al (1949) also showed that glass contact shortened the clotting time of plasma more effectively than the addition of glass macerated platelets. If cephalin was substituted for platelets the clotting time of plasma was still shortened by glass contact (Ferguson 1953). Thus the phenomenon of 'glass' contact would appear to have no direct effect on the platelets. Yet if blood is allowed to clot slowly in siliconed glass, the resultant serum is rich in prothrombin. Similarly the level of serum prothrombin is high in glass clotted platelet free plasma. These findings do suggest that both 'surface' contact and platelets are essential for normal prothrombin consumption and that they may interact at some stage during normal 'in vitro' blood coagulation.

The mechanism of glass 'contact' has been the subject of much speculation. Gortner & Briggs (1928) suggested that as glass had a negative Zeta-potential of 30 millivolts, and the same glass coated with paraffin had a sero Zeta-potential, the action of a glass surface could be due to the absorption of positively charged particles on to glass, thus selectively concentrating some blood constituent on that surface. A similar hypothesis based on electrical charges was advanced by Pickering (1928) and was revived by Wood, Horan and Sheppard and Wright in 1950. Pickering considered that the wetting of the glass by blood was sufficient to produce changes of electrical charge sufficient to inaugurate blood coagulation. These hypotheses are supported by the easily observed fact that fibrin always appears first in the blood or plasma in immediate contact with the glass surface.

Fiala & Roth (1953) considered that glass removed an inhibitor from the plasma and so allowed coagulation to proceed more rapidly. They also thought that surface had no effect on the platelets themselves. Margolis (1956) revived the suggestion that an active substance is liberated at the glass-plasma interface and afterwards destroyed by an antagonist. The actual level of activity under any given contact conditions would therefore depend on a dynamic equilibrium between these two. In his experiments he found that contact activity was independent of the number of platelets present, but a few platelets had to be present before the activity

promoted by the glass contact could be measured (Margolis 1957). Contact with glass will produce clot promoting effect in plasma, artificially rendered platelet free, and from which A.H.G., Christmas factor, factors V and VII and fibrinogen had been removed (Shafrir & De Vries 1956).

Blood platelets have been shown to adhere readily to glass. This property, apparently independent of the blood coagulation mechanism, has been extensively studied by Wright (1941, 1942, 1944, 1945, 1946 and 1951), Weiner, Zeltmacher, Reich & Shapiro (1948), Moolten & Vroman (1949), Moolten, Vroman & Vroman (1949) and Stavitsky (1953). All these workers have studied the adhesiveness of platelets in platelet rich plasma derived from both normal volunteers and patients with varying diseases. These plasma were rendered incoagulable by varying anticoagulants.

This property of platelets to adhere to glass would appear therefore, to be independent of their ability to undergo VM as, in these experiments, calcium ions were not found to be essential for platelet adhesion to glass.

<u>The Effect of Glass Surface on Viscous Metamorphosis in Native Plasma</u>

As the effect of 'glass' contact on the phenomenon of platelet VM has never been specifically observed, it was thought to be a worth while investigation. If glass contact were to have any direct effect on platelets, it was possible that platelets might undergo VM more readily in a glass tube

than in a tube with a siliconed surface. Therefore duplicate samples of the same platelet rich native plasma obtained from both normal and abnormal blood, were allowed to clot at 37°C in glass and siliconed tubes of identical size and the relationship of VM to fibrin formation observed as in Experiment 1. (Experiment 23. Appendix B, p. 18).

The results showed that glass contact did not accelerate VM, but a siliconed surface, as would be expected, delayed fibrin formation. This latter phenomenon was even more accentuated in haemophilia and Christmas disease. (Table XXIV).

The Effect of Glass Surface on the Ability of Serum to Induce Viscous Metamorphosis

The variation in the ability of normal sera derived from clotted samples of whole blood, to induce VM was difficult to explain. It has been shown that the numbers of platelets in the original blood did not influence this activity (p.50). It was possible that this difference might be the result of variation in the amount of glass contact on the donor blood received prior to clotting.

Therefore sera were prepared from native and recalcified citrate plasma, clotted in glass or in siliconed glass tubes of identical size, and their ability to produce VM determined. In addition the effect of increasing the area of glass in contact with the plasma was observed. (Experiment 24. Appendix B, p.18).

TABLE XXIV Experiment 23 Appendix B, p. 18

The effect of foreign surfaces on viscous metamorphosis in platelet rich normal and abnormal native plasma.

Duplicate samples of platelet rich native plasma (0.5 ml) were added to glass or siliconed tubes of identical size. The onset of viscous metamorphosis and fibrin formation were observed and the time sequence recorded.

Plasma	Glass Tube		Siliconed Tube	
	Start of VM in secs.	Fibrin formation in secs.	Start of VM in secs.	Fibrin formation in secs.
Normal				
1	12	230	13	359
2	110	265	120	268
3	20	226	10	236
4	30	315	35	483
Haemophilia				
5	15	525	15	1080
6	16	375	30	1210
7	25	1815	25	5400+
Christmas Disease				
8	28	460	60	1083

Glass contact was found to enhance the ability of serum to induce VM in normal washed platelets. This increased VM activity in the sera could also be measured by the increased speed at which the platelets underwent VM. (Table XXV).

Glass Surface and Thrombin Viscous Metamorphosis

The effect of thrombin on normal platelet rich citrate plasma was compared in glass and siliconed tubes of identical size. No significant difference was apparent between the two systems. This result was not surprising in view of the already rapid reaction produced by thrombin.

Glass Surface and Platelet Suspensions

In tests to measure platelet behaviour, such as platelet agglutination, it has become usual to use platelet suspensions in their own plasma,or in a synthetic medium,and to handle the blood and platelets entirely in paraffined or siliconed containers. Stefanini & Dameshek (1953) claimed that glass contact would decrease the yield of platelets or produce non-specific agglutination, and it was decided to find out whether either of their claims were true.

First it was decided to determine whether the total number of platelets in platelet rich plasma or in washed platelet suspensions was decreased on storage in glass at room temperature when compared with identical samples stored in siliconed tubes at similar temperatures (Experiment 25. Appendix B, p.19).

The effect of foreign surface on the activity of VM factor in serum.

Platelet free samples of normal native and recalcified citrate plasma were allowed to clot after contact with various areas of glass and siliconed glass at 37°C. The serum obtained was tested for VM activity against x2 washed normal platelets.

Source of Serum	Titre of VM Factor		
	Type of Surface		
	Siliconed	Glass	Glass Beads
Normal Platelet Free Native Plasma	-	1/8	1/32
Normal Platelet Free Recalcified Plasma	-	1/1	1/8

A significant decrease of the platelet count occurred when both platelet rich citrate and versene plasma were in contact with glass but not when they were in contact with silicone (Table XXVI). No drop in the platelet count occurred when washed platelets were exposed to glass.

This suggested that the platelets did adhere to the glass surface or were destroyed by virtue of some factor in the plasma or on the platelet surface that could be removed by washing.

The morphological changes induced in platelets by glass contact were observed by phase contrase microscopy. Platelet rich citrated plasma and washed platelets were added to glass and siliconed tubes and agitated continuously for 1 hour at room temperature. To increase the area of contact an equal volume of glass and siliconed beads were added to duplicate tubes and similarly agitated (Experiment 26. Appendix B, p. 19).

Glass contact was capable of inducing agglutination of many of the platelets and even produced lysis (Table XXVII). These alterations in platelet morphology were observed only in platelet rich plasma. The partial lysis in the washed platelet suspensions was thought to be the result of mechanical trauma as even silicone coated beads were capable of producing this change.

Again these observations suggest that it is a plasma factor that is activated by glass. This, in turn, was able to induce mild platelet agglutination, similar to that

TABLE XXVI Experiment 25 Appendix B, p.

The effect of foreign surface contact on normal human platelet suspensions.

1 ml amounts of normal platelet rich citrated and versene plasma and a washed suspension of normal platelets were placed in glass or siliconed tubes and agitated by rotation once every 3.5 seconds for 2 hours. Platelet counts were performed before and after rotation.

Contact Time	Platelet Counts/cu.mm. (mean of 4 counts)					
	Platelet-Rich Citrated Plasma		Platelet-Rich Versene Plasma		Washed Platelet Suspension	
	Glass	Silicone	Glass	Silicone	Glass	Silicone
0	405,000	385,000	512,000	496,000	201,000	181,000
2 hrs	221,000	409,000	385,000	478,000	197,000	209,000

TABLE XXVII Experiment 26 Appendix B, p.19.

The effect of foreign surfaces on the morphology of normal human platelet suspensions.

1 ml amounts of platelet rich normal human plasma and a x2 washed suspension of normal human platelets were added to glass and siliconed tubes and glass tubes containing glass and siliconed beads respectively. All were agitated for 1 hour by rotation once every 3.5 seconds. The morphological appearances were studied by phase contrast microscopy.

Surface	Platelet Rich Citrate Plasma	Washed Platelet Suspension
Glass	Partial lysis but no agglutination	No lysis or agglutination
Glass and Glass Beads	Partial lysis and strong agglutination	Partial lysis but no agglutination
Silicone	No lysis or agglutination	No lysis or agglutination
Silicone and siliconed glass beads	Partial lysis but no agglutination	Partial lysis but no agglutination

illustrated in Figs. 12 and 13. This agglutination in no way simulates the platelet change in VM. While it is admitted that platelets do adhere to glass, the present observations do suggest that glass contact may lyse some of the platelets and therefore any drop in the platelet numbers in suspensions in contact with glass may not be a true measure of platelet adhesiveness.

These findings do confirm the general impression that glass is injurious to platelets and that, in all experiments involving isolation of platelets or agglutination of platelets, siliconed glass wear should always be used. Yet contact must be gross before any significant change takes place in the platelets and transient glass contact, such as the accidental use of a glass Pasteur pipette, is unlikely to damage platelets or produce false agglutination.

Air

Stefanini (1955) suggested that air in the form of bubbles or the blood-air interface might also damage platelets.

Air was therefore bubbled continuously through platelet rich versene plasma and a washed platelet suspension in siliconed tubes. The platelet suspensions were prepared as described in Appendix A, p. 13. In neither sample was there any evidence of platelet agglutination or lysis.

Metal

The contact effect of a stainless steel surface on the clotting time of whole blood has been studied by Rose & Broida

(1954). They found that, while not as innocuous as silicone, it had not the same activating effect as glass and that its accelerating effect on blood coagulation was not improved by siliconing.

As many authorities have advocated coating stainless steel needles with silicone before collecting blood for the preparation of platelet suspensions, it was important to determine whether stainless steel had any effect on platelets. Therefore platelet rich versene plasma and washed platelet suspensions were agitated continuously for 2 hours in stainless steel containers (Appendix A, p. 13).

No morphological changes in the platelets of either preparation could be found. Thus it does not appear to be essential to silicone stainless steel needles before collecting blood for the preparation of platelet suspensions.

Venepuncture Technique and Tissue Thromboplastin

Any worker in the field of blood coagulation is well aware that a faulty venepuncture will shorten the coagulation time of normal and abnormal whole blood. It has been known for many years that tissue juices accelerate blood coagulation due to their content of tissue thromboplastin; the latter, together with factors V and VII, activates the conversion of prothrombin to thrombin (Biggs & Macfarlane 1953; Ackroyd 1956). Faulty venepuncture technique may introduce significant quantities of tissue juice and therefore thromboplastin into the syringe along with blood and so accelerate

the coagulation of the blood by rapid activation of prothrombin (Alexander 1955). Aynaud (1911) suggested that tissue juices might provoke platelet agglutination and lysis and Wright & Minot (1917) thought that a faulty venepuncture might produce morphologically normal platelet suspensions which were inert and quite unable to undergo VM. Yet few workers have stressed the importance of venepuncture technique when describing methods for preparing platelet suspensions from whole blood.

In the course of the present investigations several hundred venepunctures have been made in order to prepare the platelet suspensions and it was obvious that there was no factor which would destroy platelets more rapidly than the faulty venepuncture. Attempts to suck blood into a syringe whose needle is buried in tissue, produces no visible volume of fluid. But if the vein is then punctured and blood allowed to enter the syringe, it is common to find that the low spun 'native' citrate or versene plasma from such blood contains no viable platelets but only fragments of broken or lysed platelets. These fragments are quite incapable of undergoing agglutination or VM and it is possible that Wright & Minot (1917) considered these inert fragments were platelets.

It has not been possible to determine whether the platelets undergo rapid VM before they are destroyed, as no intermediate stage with large platelet clumps has been seen.

Either the plasma obtained from a venepuncture contains normal platelets or completely lysed platelets.

Tissue juice would therefore appear to contain a potent platelet lysin or VM promoting factor which was capable of breaking up the majority of the platelets before the blood could be added to an anticoagulant.

Tissue Extracts and Platelet Viscous Metamorphosis

Tissue thromboplastin is probably made up of several factors whose synergistic action measures the activity of any given tissue juice. Therefore it was possible that certain preparations might be capable of acting on platelets while others would not.

Preparations of brain prepared by differing methods, but of known thromboplastic activity, were added to platelet suspensions and the presence or absence of accelerated VM or platelet agglutination was observed (Experiment 27, Appendix B, p.20).

These fractions of human brain were found to have no action whatever on platelets either in plasma or as a washed suspension, nor did they appear to cause VM before the formation of fibrin when added together with calcium to platelet rich citrated plasma. (Table XXVIII)

Vascular Endothelium and Platelet Agglutination

It is probable that vascular endothelium, when damaged, may not only present a foreign surface to the blood stream and so induce intravascular VM, but may, by releasing some factor, be capable of directly or indirectly promoting VM (Roskam 1956).

The effect of tissue thromboplastin on platelet viscous metamorphosis.

To 0.4 ml of normal platelet rich citrate plasma or a washed normal platelet suspension was added 0.2 ml thromboplastin (brain extract) together with 0.2 ml of addition and the presence or absence of accelerated or normal VM observed.

Type of Thromboplastin	Addition	Presence or Absence of VM	
		Platelet Rich Citrate Plasma	Washed Platelet Suspension
Saline Extract of Human Brain	Saline	-	-
	M/40 $CaCl_2$	- Fibrin Clot	-
Chloroform Extract of Brain (Bell & Alton 1953)	Saline	-	-
	M/40 $CaCl_2$	- Fibrin Clot	-
Acetone Extract of Brain (Biggs & Macfarlane 1953)	Saline	-	-
	M/40 $CaCl_2$	- Fibrin Clot	-

In all instances where calcium was added together with brain extract to platelet rich plasma no VM was observed before fibrin formed. Fibrin formation was accelerated in the presence of the saline and acetone extracts of human brain.

Chance observation of vascular endothelial cells in blood films surrounded by dense masses of agglutinated platelets has supported this latter theory (personal observation). Crude saline extracts of macerated human and rabbit aortic endothelium were prepared to determine whether they could induce agglutination, lysis or VM of platelets when added to platelet rich plasma or washed platelet suspensions, with and without the addition of calcium. Neither of these extracts had any obvious effect on these platelet suspensions and they did not accelerate VM.

Russell's Viper Venom was shown by Macfarlane & Barnett (1934) to have a thromboplastin-like action of unparalleled power and required calcium for its full effect. Even when diluted many million times this coagulant action was still present. The presence of either platelets or lipoid in the plasma was essential for this action.

The direct effect of Russell's Viper Venom on platelets was studied by observing the effect of adding undiluted and diluted venom to platelet rich plasma and x2 washed platelets (Experiment 28. Appendix B, p. 20). The venom was obtained as commercial "Stypven" (Burroughs Wellcome).

Russell's Viper Venom (Stypven 1/10,000) was capable of inducing agglutination of platelets both in plasma and as washed platelets in suspension (Table XXIX). Only platelet agglutination was observed and no platelet VM or platelet lysis took place. Dilution of the venom destroyed this

The effect of adding Russell's Viper Venom to normal platelet suspensions.

Russell's Viper Venom (Stypven B & W) 0.2 ml was added to platelet rich citrated human plasma or a washed suspension of normal human platelets and the alterations in platelet morphology observed.

Dilution of Russell's Viper Venom	Platelet Agglutination	
	Platelet Rich Citrated Plasma	Washed Platelet Suspension
1/10,000	++	++
1/1,000,000	-	-
1/10,000,000	-	-
'Stypven' diluent	-	-

Russell's Viper Venom produced platelet agglutination and not viscous metamorphosis.

ability to agglutinate platelets and the commercial solvent supplied with the dried venom had, by itself, no effect on the platelets. Thus Russell's Viper Venom appears to be capable of inducing agglutination but not VM or lysis of platelets. This is thought to be a non-specific reaction unrelated to the thromboplastic activity of the venom.

The apparent destructive action of tissue juices on the platelets in blood would not appear to be related to the thromboplastic component of the tissues, but to some as yet unidentified platelet lysin.

Moolten, Vroman, Vroman & Goodman (1949) and Moolten (1953) suggested that there is a lipoid factor other than thromboplastin present in tissue fat, as well as in lymph nodes, spleen and egg yolk, which is capable of accelerating platelet agglutination and adhesiveness. This they named 'Thrombocytosin'.

Further experimental work is required to see if this lipoid agent can be isolated from fat and whether it will in fact accelerate platelet VM.

Platelet Extracts and Platelet Agglutination

It has already been shown in Chapter VI, p.65 that platelet extracts have no direct effect on either washed platelets or on platelet rich plasma.

Chylomicra and Platelet Agglutination

Following the intake of a fatty meal the blood contains numerous chylomicra or small fat particles. This is

especially marked in males who seem less capable of clearing chylomicra from their plasma than females. This increase of fat has been shown by Fullerton, Davie & Anastasopoulos (1953) and Poole (1955) to decrease the clotting time of plasma, probably by virtue of the phosphatidyl ethanolamine fraction (Robinson & Poole 1956). It was possible that this excess fat might alter platelets in some way. Therefore platelet morphology was observed both in platelet rich plasma rich in chylomicra and in washed platelet suspensions prepared from such plasma. No significant variation from normal was apparent in either preparation. This has confirmed the previous observations of Loughry & Cole (1954) who found that fat particles did not cause non-specific platelet agglutination.

Red Blood Cells and Platelet Agglutination

This phenomenon will be discussed in Part II of this thesis.

Platelet Agglutination and Animal Antihaemophilic Globulin

No discussion on platelet agglutination would be complete without describing the dramatic platelet agglutination induced in human platelets by animal preparations of antihaemophilic globulin.

Bidwell (1955 a & b) successfully produced concentrated antihaemophilic globulin from bovine and pig blood for the treatment of the haemorrhagic complications of haemophilia. This material is precipitated by selective concentrations of

phosphates from fresh citrate plasma, dialysed and subsequently preserved by lyophilisation. The A.H.G. is concentrated during this process and the activity per gramme of dried protein is equal to approximately 20 litres whole plasma. It is, however, far from pure and contains fibrinogen and probably trace amounts of prothrombin. The bovine A.H.G. fraction, when originally administered to humans by Macfarlane, Biggs and Bidwell (1954) was found to be capable of inducing massive agglutination of human platelets in vitro. Furthermore, parenteral administration of this material to a case of haemophilia produced acute thrombocytopenia in the recipient. The platelet count returned to normal levels within a few hours however.

Pig A.H.G. has not been found to have this effect (Macfarlane, Mallam, Witts, Bidwell, Biggs, Fraenkel, Honey & Taylor, 1957.)

It was decided to reassess this property of bovine antihaemophilic globulin against human platelets in the systems used in this series of experiments, namely platelet rich citrate plasma and a washed suspension of platelets.

Varying concentrations of the bovine and pig A.H.G. fractions were tested. It was arbitrarily decided to measure the concentration of the material in mgms. of dried protein. This contained active A.H.G. but this activity varied from batch to batch and can only be measured by specific assay (Biggs, Eveling & Richards 1955). The use of a given weight of dried protein is not therefore any measure

Appendix B, p.21).
of A.H.G. activity (Experiment 29). The results are recorded in Table XXX.

Platelet agglutination, in every instance when it occurred, was well established within 2 minutes.

The reaction with the bovine concentrate was so rapid and intense that it was difficult to distinguish from normal platelet VM. The platelets, however, retained their identity, no fusion appeared to occur between the individual platelets and no granules were released. It was interesting to find that although the pig A.H.G. fraction did not appear to have any thrombocytopenic effect in vivo, it did agglutinate human platelets when used in a concentration ten times greater than that of bovine A.H.G. (Table XXX).

As it was possible that these heterologous animal plasma fractions might contain a concentrated physiological platelet agglutinin, their activity was tested against both their own platelets and platelets derived from other animals (Experiment 30. Appendix B, p.21).

In none of these other platelet suspensions was there any evidence of platelet agglutination (Table XXXI). The direct specific agglutination of human platelets by bovine and pig A.H.G. would seem to represent an interspecies reaction and was not an example of the action of a concentrated physiological platelet agglutinin. Other examples of interspecies platelet agglutinins have been described by Mushett, Reissner, Weiner, Nakashima & Collett (1953), and the ability of bovine

TABLE XXX Experiment 29 Appendix B, p.21.

Animal antihaemophilic globulin concentrates and human platelets.

0.2 ml amounts of pig and bovine A.H.G. (Bidwell 1949 a & b) were added to 0.5 ml amounts of normal platelet rich citrated human plasma or a washed suspension of normal human platelets and incubated at 37°C for 15 mins. Any alteration in the platelet morphology was recorded.

Type of Animal A.H.G.	Conc. Protein mgm/ml	Presence or Absence of Platelet Agglutination	
		Platelet Rich Plasma	Washed Platelets
Bovine	0.5	+	+
	1	++	+
	5	++	+
	10	++	+
Pig	0.5	-	-
	1	-	-
	5	+	+
	10	+	+

Platelets underwent agglutination only and did not undergo the typical changes of viscous metamorphosis.

TABLE XXXI Experiment 30 Appendix B, p.21

The effect of animal antihaemophilic globulin on other heterologous platelet suspensions.

Equal volumes of animal A.H.G. and heterologous platelet rich plasma were mixed in tubes and incubated at 37°C for 5 mins. The presence or absence of platelet agglutination or viscous metamorphosis was recorded.

Platelet Rich Plasma	Bovine A.H.G. 10 mgm/ml	Pig A.H.G. 10 mgm/ml.
Pig	-	-
Bovine	-	-
Rat	-	-
Guinea Pig	-	-
Rabbit	-	-
Mouse	-	-
Cat	-	-
Dog	-	-

A.H.G. to agglutinate human platelets non-specifically supports Bergsagel's suggestion that the action of bovine thrombin on washed platelets might also be a measure of an interspecies reaction (Bergsagel 1956). (See also p. 62).

The clinical implications of this non-specific agglutination of human platelets by bovine A.H.G. has been discussed elsewhere (Macfarlane, Biggs & Bidwell 1954; Macfarlane, Mallam, Witts, Bidwell, Biggs, Fraenkel, Honey & Taylor 1957).

Summary of Chapter VII

1. Viscous metamorphosis of platelets in native plasma appears to be independent of glass contact.

2. The activity of serum VM factor is increased by glass contact.

3. Platelets were damaged by glass contact but this must be gross before any significant change occurred.

4. Air and metal surface contact did not appear to have any deleterious effect on platelets.

5. Faulty venepuncture technique invariably prevents the satisfactory separation of platelets from blood.

6. Tissue thromboplastin and vascular endothelial extracts have been shown to have no deleterious effects on platelets.

7. Russell's Viper Venom in concentrated form will agglutinate platelets but this property does not appear to be connected with its thromboplastic properties.

8. Platelets separated from plasma rich in fat or chylomicra are not apparently different from those from fat-free plasma.

9. Both heterologous bovine and pig antihaemophilic globulin concentrates were capable of producing agglutination of human platelets. They had no action on platelets separated from other heterologous species. Their action is thought to be the result of interspecies reaction.

CHAPTER VIII

Discussion

These experiments have established that, in certain experimental systems, it is possible to study, in vitro, the typical changes of platelet viscous metamorphosis (VM), and the properties of the factor or factors responsible for producing this phenomenon.

Early attempts to visualise VM as it evolved in standard microscopic preparations, were found to be unsatisfactory. The production of a thin film of platelets by the pressure of the coverslip inhibited all movement and by preventing collision between individual platelets, stopped all platelet clumping. Therefore the method of agitating the platelets in suspension in small test tubes was adopted for the study of platelet VM, and the detailed morphological changes studied by the examination of serial samples by phase contrast microscopy.

This method was thought to be justified, as when platelets start to clump and undergo VM in vivo, following damage to the vessel wall, they are travelling rapidly in the blood stream and are able to collide with each other and the damaged tissue. Thus this method approached the normal physiological process more closely than any other.

By using this technique it has been possible to demonstrate a reproducible sequence of platelet changes in normal blood and to compare these with those occurring in the

blood derived from patients with specific coagulation defects.

The morphological appearances of normal VM described and illustrated (Chapter II) were defined by studying serial samples of the varying experimental systems by phase contrast microscopy, either immediately or after fixation in formalin.

The typical changes seen when native plasma clotted could be reproduced exactly in recalcified platelet rich citrate plasma, in platelet rich citrated plasma by the addition of thrombin, or in washed platelet suspensions by fresh serum.

The clumping of the platelets, their fusion by adherence of their pseudopodia, the release of granules, and the subsequent formation of granular masses in which the individuality of the original platelets has disappeared, constitute the typical changes of VM (Figs. 1 to 11).

The striking changes that later develop in these masses after they are enmeshed in fibrin may or may not be part of VM (Figs. 9, 10, 16 and 17). Bergsagel (1956) thought that these characteristic changes, with 'balloon' formation at the periphery of the clumps, were part of VM. However, this change is not peculiar to the platelets alone and has been shown to occur in phase contrast studies of malignant cells, granular leucocytes and megakaryocytes (Zollinger 1948; Bessis 1949 & 1956). Zollinger considered that this change, which he called 'potocytosis', represented a change of the cytoplasmic gel to a sol and the resultant intake of fluid by

the cell produced the observed swelling. He thought that cells in secretory glands produced their secretion in a similar way. If his interpretation of this change is correct, these 'balloons' may represent a process of secretion of some factor by the platelets at the periphery of these clumps. If such secretion does occur, it is unlikely that 'potocytosis' has any influence on blood clotting for it does not develop until several minutes after fibrin has appeared and persists unchanged for at least 2 hours.

Bessis (1956), has claimed that potocytosis can be produced at will, by applying pressure over the cells under observation. Such pressure must always be present in phase contrast preparations of platelets as, unless they are spread out on the microscope slide, clear definition of these cells is impossible. Bessis (1954) further has illustrated potocytosis occurring in a wide variety of cells. Examples taken from his personal studies are reproduced in Figs. 19 & 20 for comparison with those changes observed in the platelets. These illustrate 'potocytosis' occurring in a megakaryocyte and a neutrophil leucocyte. These, together with the observation that similar changes can be observed in malignant cells (Zollinger 1948; Humble, Jayne & Pulvertaft 1956), suggests that this interesting cellular change is of little significance in platelet physiology and may represent cellular death, accelerated by the nature of the microscopic preparation. The intriguing differences in the 'colour' of the balloons

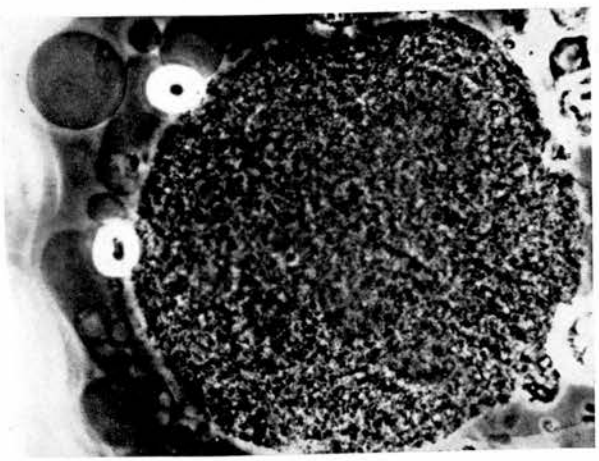

Fig. 76. — **Mégacaryocyte granuleux** dont on devine à peine le noyau au centre de la cellule. On constate la formation de nombreuses bulles exoplasmiques dont quelques-unes se sont détachées et flottent dans le liquide intercellulaire; ces bulles sont apparues après quelques minutes d'observation.

Figure 19

Potocytosis in a megakaryocyte.

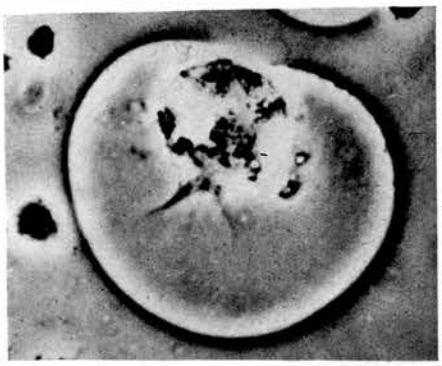

FIG. 50.

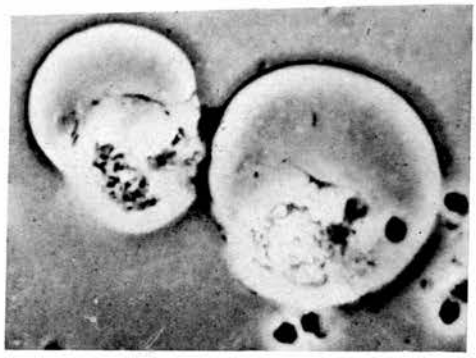

FIG. 49.

Figure 20

Potocytosis in a neutrophil leucocyte.

(Figures 19 and 20 are reproduced in this thesis by kind permission of Dr. M. Bessis and Masson et Cie., Paris.)

observed in native plasma and those in recalcified citrate plasma or thrombin clotted plasma are difficult to interpret. They are probably the result of variations in optical density of the 'balloons' or, what is more likely, of the plasma in which they are suspended. It is unknown whether this variation is of any importance.

Several workers have observed that, as VM occurs, the platelets release granular material which they thought represented the active component essential for blood coagulation (Tait & Burke 1926; Bergsagel 1956). In both normal and abnormal plasma the platelets were observed to release their granules early in the sequence of VM and, within one minute of the platelets first clumping, granules could be seen in the plasma undergoing active Brownian movement. It is thought that these granules represent that fraction of platelets essential for plasma thromboplastin formation and thus VM must occur before normal thromboplastin generation can take place.

While the experiments on platelet rich native plasma have clearly shown that VM takes place and is, in fact, fully developed by the time fibrin appears, Mann, Hurn & Mathieson (1949) and Erkelens (1956) have denied that any alteration in platelet morphology takes place in clotting blood until fibrin has formed. Basing their conclusions on the continuous microscopic observations of clotting blood, they have failed to recognise the vital function of platelet movement in the process of VM.

The present experiments have shown that at least two factors are capable of inducing the full sequence of VM prior to fibrin formation.

The first and manifestly the most important is that which produces VM within seconds of the blood being shed from the body. The time sequences and the morphological changes observed suggest that VM is one of the earliest changes of blood coagulation, and as it evolves in the presence of severe defects of antihaemophilic globulin, Christmas factor, and factors V and VII, it is probably independent of these factors. If this is so, VM must precede the initial interaction of these factors to form intrinsic plasma thromboplastin.

In Rosenthal's syndrome (Rosenthal et al 1955), where there is an apparent deficiency of yet another plasma factor (Plasma Thromboplastin Antecedent, P.T.A.) the sequence of VM differed from that observed in other syndromes. In the blood derived from three cases of this syndrome, VM did not occur until just prior to fibrin formation. This suggested that P.T.A. is in some way essential for the normal sequence of VM in the initial stages of blood coagulation. Whether it acts on the platelets directly or first reacts with another factor before producing VM is uncertain.

The Hageman factor described by Ratnoff et al (1955) may also play a part in the initial stages of blood coagulation but the exact relationship of this factor to Rosenthal's factor and VM is still unknown.

The comparison of VM in normal plasma in glass and siliconed tubes demonstrated that VM evolved normally, irrespective of surface, while the formation of fibrin was delayed in the siliconed tubes. Thus glass contact, so essential for normal blood coagulation in vitro, did not appear to influence platelet VM, but did accelerate fibrin formation. The dramatic effect of an imperfect venepuncture on the platelets did suggest that some non-haematogenous factor such as tissue juice or damaged vascular endothelium may instigate or accelerate VM. Variation in the amount of this, as yet, hypothetical factor would provide a convenient explanation for the observed variations in the time of onset and the intensity of the reaction in normals. Whether such tissue factors are in any way related to the effect of glass contact is still uncertain.

While VM appears to be independent of A.H.G. in its initial stages, it was noted that in moderate or severe haemophilia, after heparin administration and in the presence of a circulating anticoagulant, a qualitative difference from the normal existed. In all these groups the platelets clumped as in the normal and granules were released. Fibrin formation was markedly delayed in these cases, and the platelet clumps broke down and virtually disappeared before fibrin formed. This did not occur in Christmas disease, congenital factor V deficiency or induced factor VII deficiency. As heparin and the circulating anticoagulant probably inhibited

the formation of thromboplastin by preventing the utilisation of A.H.G. (Hougie 1955; Douglas 1956) it is not surprising that the same phenomena were present in these cases as in moderate or severe haemophilia. Thus A.H.G., while not essential for the initiation of VM, would appear to confer stability on the clumps.

Controversy has existed for many years on the behaviour of platelets in haemophilia and it was at one time suggested that the defect in haemophilia was inherent in the platelets themselves (Minot & Lee 1916). Many workers have claimed that either the platelets in haemophilia were more stable or clumped more slowly than in normal blood (Stubel 1914; Howell & Cekeda 1926; Tocantins 1938; Wright 1946). Yet other observers have specifically stated that they could detect no variation of platelet behaviour from the normal in haemophilia (Wright & Minot 1917; Patek & Stetson 1936; Fiala & Roth 1953; ~~Bloom 1953~~; Serf Inceman 1956). Wright & Minot (1917) did remark, however, that while VM appeared normal they noted a probable defect of "cytoplasmic integrity" in the clumps.

It cannot be said that the platelet abnormalities in haemophilia, reported in this thesis, are an explanation for these widely divergent views, but they do agree with Wright & Minot's (1917) observation. It is possible that opposing views might be formulated on platelet VM in haemophilia, if the platelets were examined at different intervals during the

process of clotting.

The occurrence of platelet VM in those cases with severe defects of A.H.G. or Christmas factor, provided evidence to refute the hypothesis that thrombin is the only factor capable of producing VM (Quick 1951; Quick & Hussey 1952; Stefanini 1951; Luscher 1956; Bounameaux 1955). It is inconceivable that any thrombin could be generated in such cases within seconds of the start of coagulation (see Table I).

The failure of heparin, a potent anti-thrombin, when used in weak concentrations just capable of preventing fibrin formation, to prevent VM confirms the results of Baranovsky & Quick (1943) and ~~also~~ Jurgens & Braunsteiner (1950) and provides evidence to negate the hypothesis that VM in the early stages of blood coagulation is always due to trace amounts of thrombin. It also tends to confirm the assumption that platelet VM in native plasma is identical to that seen in a damaged blood vessel in vivo as Best, Cowan & Maclean (1938), Solandt & Best (1940) and Zucker (1947) have ~~clearly~~ shown that, while heparin in large concentrations inhibited all platelet VM in damaged vessels or in artificial arteriovenous stunts, it would not, when used in small concentrations.

Calcium is essential for normal VM, although only trace amounts, insufficient for normal fibrin formation need be present.

Soya bean trypsin inhibitor, a known inhibitor of active

thromboplastin (Macfarlane 1947) did not inhibit VM. Conversely Thrombodym (Neodymnium 3-sulpho-iso-nicotinate) in large concentrations sufficient to inhibit or delay fibrin formation also inhibited VM. The anticoagulant action of this drug has been described by Hunter & Walker (1956), who found that this drug inhibited Christmas factor and factor X and, in large doses, A.H.G. They did suggest its action might lie in an antimetabolite action on calcium and, if correct, this could explain its inhibitory effect on VM.

It has not yet been established whether this drug has any effect on the P.T.A. factor of Rosenthal.

In native plasma derived from cases of idiopathic thrombocytopenic purpura and in artificial thrombocytopenic plasma no platelet VM took place. From the experiments using washed platelet suspensions and active fresh serum (p.51) it was found that platelets must be present in a density of at least 60,000/cu.mm. before VM can evolve normally. This finding will be discussed further in relation to the mechanism of haemostasis.

While it appeared unlikely that thrombin influenced platelets in the early stages of blood coagulation, it was found that human thrombin, prepared by the method of Biggs & Macfarlane (1953) could, in fact, induce platelet VM when added to platelet rich plasma. This could only be seen when small amounts of thrombin were added as the rapid formation of fibrin obscured the platelet reaction when large concentrations were used. When small concentrations of

thrombin (0.5 u/ml) were added, VM evolved very rapidly developing approximately 60 seconds prior to fibrin formation and, after the fibrin had appeared, the subsequent evolution of VM was quicker than that seen in normal plasma. This rapid sequence suggested that in normal physiological coagulation thrombin may induce platelet VM, but only at a stage just prior to fibrin formation. This finding agreed with the observation that VM was apparently augmented in clotting platelet rich plasma just prior to the fibrin appearing. Human thrombin, while capable of inducing VM in platelet rich plasma, had no influence on washed platelets. Its VM activity was therefore dependent on a co-factor in the plasma or adsorbed onto the platelets. It has been shown that this could not be P.T.A., A.H.G., Christmas factor, factors V or VII, prothrombin or normal fibrinogen, but that it did appear to be some factor, as yet undetermined, loosely bound to the platelets themselves and easily separable from the latter by washing or storage in vitro. Boumameaux (1957b) has suggested that this thrombin co-factor is not one factor but a complex mixture consisting of glucose, phosphate, ester phosphates of glucose and fructose. This opinion still has to be confirmed but his observations on the behaviour of this complex co-factor do not agree completely with the present observations.

The changes induced by bovine thrombin on washed platelets appear to be those of simple agglutination rather than

typical VM; a finding which supports Bergsagel's (1956) view that this was an example of a non-specific interspecies reaction.

The experiments discussed in Chapter VI have suggested that the factor which Bergsagel (1956) claimed to be capable of inducing VM in washed platelets was not, as he thought, an intermediate product of thromboplastin formation, but thrombin. The mixtures used by Bergsagel in his experiments were capable of generating thrombin prior to the addition of platelets. This opinion does depend on the assumption that his materials also contained co-factor activity as he used washed platelets as his indicator. That pig A.H.G. does have this activity has been shown on p.64 The physical properties of the VM factor generated in similar experiments were similar to those of thrombin and thus provided further evidence in support of this opinion.

The factor in fresh serum which is capable of inducing VM in washed platelets in many ways resembles thrombin. Like thrombin the serum VM factor is inhibited by heparin even in small doses, but not by Thrombodym or soya bean trypsin inhibitor. Unlike thrombin serum VM factor did require calcium before it could act and it could induce VM in washed platelets. Stefanini & Silverberg (1951) and Luscher (1956) have suggested that when platelets are added to fresh serum the unconsumed prothrombin is converted to thrombin which in turn induces VM in the platelets. Attempts to

Attempts to demonstrate this generated thrombin by the addition of fibrinogen to platelet-serum mixtures failed completely. If this VM property of serum was due to trace amounts of thrombin, insufficient to clot pure fibrinogen, it is difficult to see how this could be proved. Thrombin can only be defined as that substance which will convert fibrinogen to fibrin. To assume any other activity must be pure hypothesis until the biochemical nature of thrombin can be determined.

The activity of the VM factor in serum was not influenced by the absence of P.T.A., A.H.G., Christmas factor, factors V and VII from the blood from which it was derived; similarly the number of platelets in the donor blood did not influence its activity. The amount of glass contact imposed on the original blood or plasma prior to clotting did, however, influence the VM activity of serum.

By using the ability of fresh normal serum to induce VM in platelets it was possible to show that platelets derived from cases of haemophilia, Christmas disease, P.T.A. deficiency (Rosenthal's syndrome), congenital factor V deficiency and acquired factor VII deficiency were capable of normal VM.

While it has been assumed for the purpose of these experiments that the human thrombin used in these experiments is pure, it is by no means certain that this is so. The method used to separate it from plasma by primary precipitation

of the prothrombin carrying fraction, recalcification, subsequent precipitation by acetone and elution of the thrombin by saline may not be specific and it is remotely possible that such preparations may contain some contaminant which, by itself, is capable of inducing VM in platelet rich plasma. Therefore the VM which follows the addition of thrombin may only be incidental to the ability of the material to convert fibrinogen to fibrin. This doubt cannot be removed until further experiments have more clearly elucidated the nature of the VM factors in the inital stages of coagulation or in serum.

It would be convenient to find a theory which would reconcile the differences in the factors influencing VM in the three systems examined in these experiments (Table XXIII). These differences, however, cannot be explained in terms of modern theory, and it must be assumed that three separate factors may induce VM in platelets either before or after the blood clots. It is reasonable to propose that during blood coagulation platelets would appear to undergo VM in the initial stages by the action of a factor which may be either P.T.A. or a factor produced by disruption of extravascular or vascular tissue or both. This process releases the platelet thromboplastic component, which, reacting with A.H.G., Christmas factor and calcium, forms an intermediate product which, reacting with factors V and X, forms active thromboplastin; this converts prothrombin to thrombin. The latter

then, would appear to augment the action of the primary factors and accelerate the process of VM in those platelets which until this time have not become involved. This theoretical sequence does fit in with the observed morphological evolution of VM. The apparent accentuation of the reaction just prior to fibrin formation in cases with P.T.A. deficiency and haemophilia, would strongly support this secondary effect of thrombin. Once the blood has clotted the expressed serum can further promote VM. This latter function, while apparently of rather academic interest in 'in vitro' studies, may play a most important role in intravascular haemostasis and thrombosis. The ability of fresh serum to promote VM, may explain why serum can rapidly clot platelet rich citrate plasma and the observed power of serum to induce intravascular thrombosis when injected intravenously (Wessler 1953).

Perhaps the most significant finding in these experiments has been the definite interval that was observed to elapse between the development of definite VM and fibrin formation. This interval, especially marked in severe haemophilia and Christmas disease, in the presence of a circulating anticoagulant, and following the administration of heparin, may even be significant in normals.

From the many 'in vivo' studies it is almost certain that platelet VM plays an important role in the mechanism of haemostasis. When a vessel is damaged or partially cut the

platelets accumulate over the damaged section within seconds of the wound being inflicted or form a plug around and in the breach in the wall. The factor or factors responsible for this rapid reaction on the part of the platelets have still to be determined.

If the blood flow in the vessel is maintained it has been noted that no fibrin forms around these platelet masses. But fibrin formation may occur if the blood flow ceases by virtue of vaso-constriction or complete occlusion of the vessel by the platelet mass.

The fact that platelet thrombi can form without fibrin formation was first noted by Eberth & Schimmelbusch (1888), and the observed interval between VM and fibrin formation may provide an explanation for this phenomenon. As it is thought that the platelet products, necessary for thromboplastin formation, are released early in the sequence of VM, to take part in a time-consuming reaction with A.H.G., Christmas factor, calcium and factor V, it is not unreasonable to suppose that, if blood is still flowing past the platelet mass, the platelet products can be washed away or diluted before they can react with these other factors. Thus the platelet mass may be left in situ, being reinforced as other platelets collide with it, but without any fibrin formation around it. Theoretically a thin layer of fibrin may form on the surface of the platelet mass where escaping products tend to be concentrated, or in the interstices of the clump. If

the blood becomes static in the vessel or by extravasation, no such dilution factor will apply and the platelet products will react with the other coagulation factors in situ and fibrin will be formed. Admitted hypothetical, this theory does agree with the views of those workers who have observed the mechanism of haemostasis in animals and humans.

If true for normal blood, this interval between VM and fibrin formation is even more significant in those cases where a defect of one coagulation factor has significantly delayed fibrin formation and so prolonged the interval. In severe haemophilia and Christmas disease, the difference from normal is so marked that it must play some part in the defective haemostasis in these syndromes. In both, normal primary haemostasis appears to follow trauma, but after a variable interval, severe secondary haemorrhage may occur and persist for days and even weeks. The following theory can be advanced to explain this not uncommon occurrence. The platelets, undergoing VM in the initial stages of clotting, form a haemostatic plug and release both their coagulation products and vaso-constrictive agents; thus the initial flow of blood from the vessels is stemmed. At present it is unknown how long the platelet mass or vaso-constriction may persist in humans, but it is entirely possible that one or other may disappear before the platelet products have had time to react with the defective coagulation system to form reinforcing fibrin. It is even possible that, in severe haemophilia and Christmas

disease, fibrin formation may be delayed or imperfectly formed in static or extravasated blood. If this is so, the failure of the immediate haemostatic mechanism unsupported by normal fibrin formation will allow profuse secondary haemorrhage to occur. Why the process of platelet agglutination and vaso-constriction cannot be repeated, and so stop this secondary blood loss, is unknown. Yet, if Margolis' (1957) observations of the effect of glass contact on blood coagulation are applicable to damaged tissue surfaces, it is probable that the effect of the latter is also capable of exhaustion and thus has only a transient effect on the blood coagulation.

The apparent instability of the platelet clumps in haemophilic blood may provide an explanation for the observed clinical differences between Christmas disease and haemophilia. In the former, a virtual complete absence of Christmas factor is not usually accompanied by such a severe haemostatic defect as that seen in classical haemophilia where no antihaemophilic globulin can be determined. The total absence of VM in thrombocytopenic blood was supported by the experimental observation that at least 60,000 platelets must be present before normal VM can occur (Chapter V). The haemorrhagic tendency associated with thrombocytopenia cannot be due solely to this failure of VM as, in some patients with very few circulating platelets, no haemostatic defect can be determined. ~~(Chapter II, p.).~~

Again it must be stressed that this analysis of the mechanism of haemostasis in the light of the experimental results of this thesis, is purely hypothetical, but it cannot be denied that it has provided an explanation for some of the discrepancies encountered in studies of normal and abnormal haemostasis.

With this knowledge it is intended to carry out further research to determine whether these assumptions can be substantiated by in vivo studies. Such an investigation will necessitate employing histological as well as vital studies in both experimental animals and humans. So far, orthodox histological techniques have failed to differentiate fibrin from viscous metamorphosed platelets. As can be seen from the illustrations of platelet changes in VM in Chapter II, this is not entirely surprising, as it is difficult to recognise the platelets as such once they have clumped. Thus the solution of this problem must await the development of new techniques or the adaptation of established methods.

The influence of glass contact on platelets is difficult to assess. From published experimental work it is obvious that glass contact will quantitatively accelerate normal blood coagulation and that some degree of glass contact is essential for what is accepted as normal 'in vitro' coagulation. While glass does not seem to have this action by virtue of damaging platelets, but rather by activating some plasma component, there is no doubt that glass contact will damage platelets. These cells will adhere to glass ~~when~~

when suspended in their own plasma and will in fact agglutinate when exposed to a large area of glass for a long period. They do not, however, undergo the typical sequence of VM.

While VM in platelet rich native plasma appeared to be independent of glass contact, evolving normally in siliconed tubes, it cannot be denied that the bloods examined may have received some contact during the process of collection prior to testing. This contact could not have been due to glass but it is possible that damaged tissues may have similar properties to glass surfaces.

The observed increased activity of the serum VM factor by glass contact does suggest that this factor could be the actual 'contact factor' as described by Margolis (1957) and Shafrir & De Vries (1956). This supposition is strengthened by the failure to relate this serum VM factor to any known coagulation factor.

Contact with air or a stainless steel surface did not appear to have any deleterious effects on the platelets.

No factor could damage platelets more dramatically than bad venepuncture technique. Whether this is due to some as yet undetermined tissue platelet lysin or was simply an expression of accelerated coagulation by tissue thromboplastin has not been decided. Experimentally tissue thromboplastin had little or no effect on the platelets either during coagulation or when added to platelet suspensions.

The effect of Russell's Viper Venom, a potent thromboplastin, must be assumed to be an example of non-specific platelet agglutination rather than an expression of the coagulant activity of this material.

The ability of both bovine and pig A.H.G. to agglutinate platelets, while apparently irrelevant to the present problem, provides yet another example of non-specific platelet agglutination. The recording of these observations is worth while, however, as they do emphasise the dangers of using heterologous proteins in blood coagulation research, especially where platelet systems are being employed. It is of interest that this effect appears to be peculiar to human platelets only, although not all animal platelet suspensions have been examined.

The experiments so far recorded and discussed have provided information of value to the understanding of normal blood coagulation and haemostasis. They have also clarified the original problem that presented while trying to demonstrate platelet agglutinating factors, other than those concerned in the process of blood coagulation, in the blood of patients with idiopathic thrombocytopenic purpura.

Thus it is now possible to proceed to the second part of this thesis which constitutes an investigation into the validity of the many claims to have demonstrated platelet agglutinating factors in the blood of patients with thrombocytopenic purpura and associated syndromes.

PART II

CHAPTER IX

A Brief Historical Review of the Pathogenesis of Thrombocytopenic Purpura with Special Reference to the Theory of Immune Mechanisms

Cutaneous purpura has been recognised since the 15th century but Werlhof (1776) is, by custom, credited with the first description of the clinical appearances of idiopathic or essential purpura. He called the syndrome "morbus maculosus haemorragicus" to describe the association of cutaneous purpura with epistaxes and menorrhagia. But it was not until the last two decades of the 19th century that platelets were recognised as separate cellular elements of the circulating blood and were found to be reduced in number in certain types of purpura (Krause, 1883; Denys, 1887; Hayem, 1895).

The classical clinical features and laboratory findings of idiopathic thrombocytopenic purpura were described fully by Duke (1912) and these remain unchanged to-day.

Once Bizzozzero's (1882) belief that platelets were separate elements of the circulating blood, and not fragments of broken down leucocytes or red cells (Howell, 1891), precursors of the latter (Hayem, 1878), or precipitate plasma proteins (Donnée, 1842), and their origin from the megakaryocytes was accepted (Wright, 1910), theories began to be propounded to explain why the platelets disappeared from the circulation in thrombocytopenic purpura.

The thrombocytopenia, with associated purpura, that accompanied the destruction of the marrow and the megakaryocytes by poisons or by replacement with neoplastic, leukaemic or fibrous tissue, has been easily understood and accepted (Minot, 1917). On the other hand, the idiopathic or essential variety of thrombocytopenic purpura, appearing suddenly and either disappearing spontaneously, or persisting for months or even years, had attracted experimental workers and the theorists for many years.

The recognition that immune mechanisms might be a partial or complete explanation for this syndrome is not as modern as the present literature would lead the reader to believe.

As long ago as 1896, Hayem, in one of his excellent papers on platelets ("les hématoblasts") described "la pénétration dans le sang d'une substance altérant les hématoblasts et les précipitants. Ce sont les hématoblasts altérés qui reunis en amas, seraient la cause des hemorragies et mieux des infarctus hemorragiques". He did not qualify this remarkably prophetic statement but the standard of Hayem's observations was so high that it is probable this was the result of experimental observation. Thus he must have been the first to describe a platelet agglutinin.

Very rapidly it was found that, if an animal was injected with platelets derived from a heterologous species, antisera could be prepared which would agglutinate the platelets of

immunising species (Marino, 1905). Anti-human platelet serum produced in rabbits was capable of agglutinating and lysing human platelets, even when diluted (Cole, 1907). It would agglutinate human red cells only when used undiluted and this differential agglutination tended to confirm Bizzozzero's hypothesis of platelet individuality from other cells of the blood. Similar antisera were found to be capable of producing the full clinical picture of purpura as well as thrombocytopenia (Ledingham, 1914; Ledingham & Bedson, 1915; Lee & Robertson, 1916; Leonard & Falconer, 1941). Their action was specific for the platelets of the species used to immunise the antisera donor animals. Lee & Robertson (1916) noted that normal sera, which did not contain any antibodies, were also capable of inducing transient thrombocytopenia but never purpura, when injected into animals of a heterologous species.

Bedson (1921, 1922) reported a series of important experiments in which he showed that, while purpura always accompanied the thrombocytopenia induced by heterologous anti-platelet sera, it did not appear if the thrombocytopenia was induced by mechanical methods (intravenous injection of 0.5% agar in serum). When he injected anti-red cell serum prior to depleting the animal of platelets by agar-serum, purpura developed. Thus he demonstrated in animals what has so often been observed clinically in humans, namely, that thrombocytopenia

can be present without concomitant purpura developing. Splenectomy was found to protect animals from the effect of anti-platelet sera (Bedson, 1924) but Elliot and Whipple (1940) found that abnormal capillary fragility could still be demonstrated if very large doses of platelet destroying antisera were given to splenectomised animals.

At this time, experimental work started in Japan by Katsura (1923, 1924), showed that non-thrombocytopenic purpura could be induced by heterologous antisera prepared against vascular endothelium. This serum, however, when used along with anti-platelet sera, enhanced the effect of the latter and produced ~~severe~~ thrombocytopenia in the animals. By Complement fixation he thought that he could demonstrate that platelets and vascular endothelium shared at least one common antigen. These observations were unknown to the Western world until Clark & Jacobs (1950) translated the original papers and confirmed the Japanese observations.

Tocantins (1936a) has stressed the ability of anti-platelet sera to produce a rapid fall of the blood platelet level within minutes of injection. The platelet count returned to normal levels within a few hours. Leonard & Falconer (1941) felt that the concomitant purpura was found only when the platelet count dropped rapidly and not if the thrombocytopenia developed slowly.

This growing realisation that thrombocytopenia alone could not explain all the manifestations of idiopathic purpura led

others to stress that capillary damage must play an important role in the pathogenesis of purpura (Bedson, 1922; Tidy, 1926; Elliot & Whipple, 1940; Robson, 1949; Clark & Jacobs, 1950). Altered capillary function in thrombocytopenic syndromes was noted by Macfarlane (1941).

It is surprising that these experiments did not stimulate a more rapid enquiry into the occurrence of platelet agglutinating or lytic factors in the blood of cases of idiopathic thrombocytopenic purpura in humans. Lee & Robertson (1916) did think of the possibility but obtained negative results in the one case they examined.

One explanation for this may have been the preoccupation of other workers in the role of the spleen in this syndrome. Following the reported success of splenectomy in the treatment of idiopathic thrombocytopenic purpura (Kaznelson, 1916; Hess, 1917; Myers, Maingot & Gordon, 1926), many believed that the spleen generated a humoral factor capable of destroying platelets or inhibiting their formation from megakaryocytes (Minot, 1917; Wiseman, Doan & Wilson, 1940; Dameshek & Miller, 1946).

The isolation of a factor from the spleens of thrombocytopenic patients, capable of inducing thrombocytopenia when injected into experimental animals, strengthened the beliefs of these workers. This was named 'Thrombocytopen' by Troland & Lee (1938) and Otenasek & Lee (1941). Their findings were confirmed by Torrioli & Puddu (1938) and

Hobson & Witts (1940), but both groups remarked that the method of extraction was all important as normal splenic extracts were also capable of this action. Torrioli et al (1938) thought that the main action of these extracts was to damage the megakaryocytes. A further possible function of the spleen was advanced by Holloway & Blackford (1924) and Wiseman, Doan & Wilson (1940) who, in a comparative study of platelet counts in the splenic artery and vein, noted that the count was lower in the vein. This led them to propose that selective "sequestration" of the platelets occurred in the spleen and that any over-activity would lead to thrombocytopenia. This sequestration theory has been denied by Stefanini, Chatterjea, Dameshek, Welch & Swenson (1952), but Bell & Alton (1955) have again claimed to have shown selective sequestration of platelets by the spleen in cases of idiopathic thrombocytopenia, but not in normals.

Accordingly two concepts of pathogenesis developed. One assumed that increased platelet destruction constituted the main basic defect while the other supported defective production. The observation that anti-platelet sera also produced morphological changes in the megakaryocytes and so might influence the manufacture and release of platelets unified the two theories (Tocantins & Stewart, 1939; Pisciotta, Stefanini, & Dameshek, 1953). The unique common antigenicity of the megakaryocytes and the platelets has been confirmed by the use of fluorescein labelled anti-platelet serum (Humphrey, 1955).

The modern era of research into the role of immune mechanisms in the pathogenesis of idiopathic thrombocytopenic purpura was stimulated by Evans & Duane (1949) who drew attention to the not infrequent finding of low blood platelet levels in cases of acquired haemolytic anaemia where anti-red blood cell antibodies were demonstrable in the circulating blood of the affected patient. The natural history of these cases, they stressed, was very similar to that of idiopathic thrombocytopenic purpura and they postulated that the latter syndrome could be the result of a similar mechanism. They proceeded to confirm their hypothesis by demonstrating platelet agglutinating agents in the blood of cases of thrombocytopenia (Evans, Takahashi, Duane, Payne & Liu, 1951). Almost simultaneously Ackroyd reported that, when he added the drug 'Sedormid' to the blood derived from patients with 'Sedormid'-induced thrombocytopenic purpura, he was able to show in vitro platelet agglutination and lysis and, by similar means, inhibit all clot retraction after clotting had occurred (Ackroyd, 1949 a, b, c). He was also able to show that Complement was fixed during this reaction and that this occurred only in the presence of the specific drug 'Sedormid' (Ackroyd, 1951). These findings demonstrated clearly an immune mechanism in drug induced purpura and supported the Evans-Duane hypothesis.

Further supporting evidence was rapidly produced by transfusion studies in cases of idiopathic thrombocytopenic

purpura. It has been established that the natural survival time of the platelet in animal and human blood varied between 5 and 9 days when studied in vivo by radio isotope techniques (Odell, Tausche, Furth, 1954, 1955; Leeksma & Cohen, 1955, 1956). Survival was shorter by 2 - 3 days if the platelets were removed from the body, tagged with the isotope, and retransfused (Odell et al, 1954, 1955). When polycythaemic platelet rich blood was transfused by a direct method using a siliconed syringe into patients with various forms of thrombocytopenia, it was possible to raise the platelet count to 90,000/cu.mm. and normalise the haemostatic function as measured by the bleeding time, prothrombin consumption index, and vascular fragility. These platelets survived for 4 - 6 days in secondary thrombocytopenia but in idiopathic thrombocytopenic purpura, especially the acute variety, they only survived for 30 mins. - 24 hours, indicating a destructive process existed (Hirsch & Gardener, 1951,1952; Stefanini, Chatterjea, Dameshek, Zannos & Santiago, 1952; Sprague, Harrington, Lange & Shapleigh, 1952).

The apparently dangerous experiment of transfusing blood from cases of idiopathic thrombocytopenic purpura into normals produced dramatic falls in the platelet counts of the recipients and, in some, overt purpura. This induced thrombocytopenia persisted for several days (Harrington, Minnich, Hollingsworth & Moore, 1951). In a further series of 35 cases, Harrington and his co-workers demonstrated that

they were able to induce thrombocytopenia in normals by injecting blood from 16 separate cases of idiopathic thrombocytopenic purpura (Harrington, Sprague, Minnich, Moore, Aulvin & Dubach, 1953). Identical results were obtained using the blood of two such cases following a return of their platelet counts to normal after successful splenectomy. Harrington's observations were confirmed by Stefanini, Dameshek, Chatterjea, Adelson & Mednicoff (1953), and Kissemeyer Nielsen (1953b).

These findings were parallel with those obtained following the injection of heterologous anti-platelet serum in animals and therefore provided strong evidence that idiopathic thrombocytopenic purpura was due to the presence of a circulating anti-platelet factor in the blood.

This apparently irrefutable evidence has been somewhat modified by the observations of Stefanini & Chatterjea (1952), who claimed that normal blood or plasma when transfused into normals could induce transient thrombocytopenia. Purpura, however, did not result. Serum they found produced a more sustained reaction. Further, Krevans & Jackson (1955) found that, if large volumes of stored blood were transfused rapidly into normals, thrombocytopenia and even purpura, together with impairment of haemostatic function, would ensue. Both series stated that splenectomised normals reacted in a similar fashion. Also, it was shown that exchange transfusion of the newborn induced thrombocytopenia (Stefanini, Mednicoff,

Salmon & Campbell, 1954; Desforges & O'Connel, 1955). A somewhat confusing observation was made by Wilson, Eiseman & Chance (1952) who claimed that when they repeated Harrington's experiments the induced thrombocytopenia was apparent in the recipient only when they measured the platelet count by the indirect method of Dameshek (1932) (numbers of platelets assessed by comparison with the total number of red cells/cu.mm. of blood) and not when they used direct methods (directly counting the platelets, after lysis of red cells, in a counting chamber). The significance of their findings is obscure and they have not been confirmed. The most likely explanation for this post transfusion thrombocytopenia would appear to be the diluting effect of transfused stored blood and the non survival of the donors platelets in the recipients circulation. Thus these findings do not necessarily detract from the importance of Harrington's findings.

Stefanini, Dameshek & Adelson (1952) observed that, if repeated transfusions of fresh blood were given to thrombocytopenic patients with aplastic anaemia, the survival time of the transfused platelets in the recipient's blood became shorter and the clinical improvement less obvious after each transfusion. On the assumption that these repeated transfusions might have induced the formation of a platelet agglutinin in the recipient, blood from eleven patients who had received multiple transfusions were tested

but in only one was a platelet agglutinin found. A similar decrease of the beneficial effect of repeated platelet transfusions was reported by Hirsch & Gardener (1952) who also felt that platelets administered by transfusion might be antigenic. Both Harrington and Stefanini and their co-workers found further evidence to suggest that repeated platelet or whole blood transfusions were antigenic and had produced platelet agglutinins in the recipients (Harrington et al, 1953; Stefanini, Plitman, Dameshek, Chatterjea & Mednicoff, 1953).

Simultaneously with these observations, Sprague, Harrington, Lange & Shapleigh (1952) and Harrington et al (1953) were able to demonstrate that platelet agglutinins could be demonstrated in the blood of cases suffering from idiopathic thrombocytopenia. These experiments together with early observations of Evans et al (1949 & 1952) stimulated other workers to explore this field and to report the presence of platelet agglutinins in similar patients (Stefanini, Chatterjea & Adelson, 1952; [illegible]; Dausset, Delafontaine & Fleuriot, 1952; Miescher, Vanotti, Couchaud & Hemmeler, 1952; Nolthenius, Hoorweg & Van Loghem, 1953; Dausset, 1954; Verstraete & Vandenbroucke, 1955; Weinreich, 1955, 1956).

One isolated case was described by Stefanini and his co-workers which is worth more detailed analysis (Stefanini, Dameshek, Chatterjea, Adelson & Mednicoff, 1953). In this case they were able to demonstrate a powerful platelet agglutinin with a titre of 1/2048 by direct agglutination

tests. When blood from this patient was transfused into a normal individual, not only did it produce severe thrombocytopenia in the recipient which persisted for 12 days, but a measurable agglutinin could be detected in the recipient's blood. This agglutinin disappeared only as the platelet count returned to normal. The megakaryocytes in the marrow of the normal recipient were also damaged and the platelets gave a positive direct reaction when tested with anti-human globulin serum (Coomb's Test) (see p.118). A study of the physical properties of the platelet agglutinin revealed that it was a relatively well defined factor. One unusual property was the absorption or neutralisation of this agglutinin by guinea-pig kidney. This property is peculiar to Forssman or heterophil antibody and although Forssman type antibodies have been detected in the sera of patients with idiopathic or secondary thrombocytopenic purpura in a titre higher than that found in normal sera (Stefanini & Adelson, 1952), these sera, unlike the case of Stefanini et al (1953), did not agglutinate human platelets (Adelson & Stefanini, 1952).

Electrophoretic separation showed that this high-titre agglutinin was apparently in the beta-2 globulin fraction (Stefanini et al, 1953) yet the platelet agglutinin or lysin detected by Tullis (1953a, 1956) appeared to be confined to the gamma globulin fraction as did the antibody measured by Stefanini on an earlier series (Stefanini, Chatterjea & Adelson, 1952 b)

Tullis claimed that he was able to detect a platelet lysin in the sera from patients with idiopathic thrombocytopenia. He was able to demonstrate a fall in the non-agglutinated platelet count after incubation of a mixture of patient's serum and normal washed platelets. His claim has received support from Bessis & Tabuis (1955) who also were able to show platelet lysins in the presence of Complement in blood from cases of idiopathic thrombocytopenia.

All these series, using differing techniques, show considerable variation in the number of cases of idiopathic thrombocytopenic purpura in which platelet agglutinins could be detected and in their distribution of positive tests between acute and chronic varieties. The techniques used and the distribution of positive results are summarised in Table XXXII, at the end of this chapter.

The failure of the direct platelet agglutination test to detect agglutinins in every case prompted other workers to explore other techniques in the quest of a more sensitive and reliable method for detecting anti-platelet factors in blood of cases of idiopathic thrombocytopenic purpura.

The adaptation of the Coomb's test (Coombs, Mourant & Race, 1945) to this problem was an obvious step. This test was devised to detect incomplete or non-agglutinating immune anti-red cell antibodies lodged on the surface of red cells. As these antibodies were globulins, the addition of anti-human globulin rabbit serum, agglutinated these red cells. It

was probable that if immune antibodies existed in the blood of patients with idiopathic thrombocytopenic purpura, some might be incomplete and coat the platelets without producing agglutination, and therefore might be demonstrable only by using Coombs' technique. Fluckiger, Hassig & Koller (1953) found that, in 5 cases of idiopathic thrombocytopenic purpura where no platelet agglutinins could be demonstrated by direct agglutination techniques, 3 positive direct Coombs' tests were obtained when anti-human globulin serum was added to washed suspensions of the patient's platelets. Considerable doubt was thrown on their results by Sauer & Van Loghem (1954) who tested 47 cases of idiopathic thrombocytopenic purpura using this technique with completely negative results. Dausset & Malinvaud (1954) found that certain anti-human globulin sera would agglutinate all platelet suspensions, normal and otherwise. To add to the confusion, Nelken & Gurevitch (1956) have claimed that in 2 cases of acquired haemolytic anaemia due to immune antibodies, the Coombs' test was positive with both the red cells and the platelets. Both cases, however, had normal platelet counts and did not show any abnormal bleeding tendency.

The indirect Coombs' test, used to detect the presence of an incomplete antibody in serum has been adapted by Jaeger-Draefsel, Weigman & Van Loghem (1956) to test the sera from cases of idiopathic thrombocytopenic purpura with apparently encouraging results. Preliminary incubation of

these sera with normal human platelets was performed to allow any non-agglutinating anti-platelet factor in the sera to coat the platelets. The subsequent addition of anti-human globulin serum to these platelets proved that such antibodies were present in many of these sera.

Moulinier (1955) proposed a further modification of the indirect Coombs' test by comparing the titres of residual activity in anti-human globulin serum after exposure to platelets sensitised by preliminary incubation with serum from cases of idiopathic thrombocytopenic purpura and normal serum. He claimed that this was a far more sensitive technique than the classical indirect Coombs' technique.

Kissemeyer Nielsen (1953a) devised an ingenious technique for measuring anti-platelet factors, based on a modification of Boyden's tannic-acid-red cell-protein indicator for measuring anti-protein sera (Boyden, 1951). After first incubating washed human red cells with tannic acid, he exposed them to a human platelet protein extract which was absorbed onto the tanned red cell surface. These coated red cells were added to test sera and, if the former were agglutinated, these sera were throught to contain anti-platelet antibodies. This technique gave reproducible results with anti-human platelet rabbit serum and suggestive results with sera derived from some cases of idiopathic thrombocytopenic purpura. This technique was adopted by other workers who supported Kissemeyer Nielsen's

claims (Sauer & Van Loghem, 1954, 1955; Vandenbroucke & Verstraete, 1955; ~~Dausset, 1955~~).

Ackroyd (1955a) however, reported dissatisfaction with this technique and Dausset, achieving a partial volte-face, now claims that this technique, far from measuring platelet antibodies, appears to detect some unknown substance released from fibrin after fibrinolysis has occurred (Dausset, Bergerot-Blondel & Paraf, 1956).

Dausset (1953) and Nicola, Rosti, & Zangaglia (1955) attempted to determine whether Complement was fixed when serum from cases of idiopathic thrombocytopenic purpura were incubated with normal platelets but, in both series, entirely negative results were obtained.

Jamra, De Angelis, Verrastro & Coelho (1953) devised a technique to measure platelet lysins similar to that used by Tullis (1953a & 1956) but instead of counting the number of non-agglutinated platelets before and after exposure to test sera, these workers estimated the packed platelet volume (thrombocrit). They claimed that they were able to show a drop of 50% in the volume of platelets, after exposure to serum from one case of thrombocytopenia. Nolthenius, Hoorweg, Van Loghem (1953) found what they considered to be platelet agglutination promoting factors, in serum of certain cases of thrombocytopenic purpura. They found that when they added test serum from 3 cases of purpura to anti-human platelet rabbit sera the titre of the latter was increased

by 100 - 500%. Of their 3 cases only one was thrombocytopenic. They rightly considered that this phenomenon of agglutination promotion was analogous to that measured by the Differential Agglutination Test used in the diagnosis of cases of rheumatoid arthritis (Rose, Ragan, Pearce, & Lipman 1948). Yet another technique to demonstrate anti-platelet factors in certain sera has been suggested by Moulinier & Mesnier, (1954). They first prepared a lipoid extract of platelets with alcohol, ether, and chloroform which, when added to a suspension of micro crystals of cholesterol, provided sensitised particles that could be agglutinated by certain sera from a mixed group of patients with thrombocytopenia but not by normal sera. So far there has been no confirmation of the efficacy of this test.

Before completing this survey it is necessary to consider two further aspects of thrombocytopenic purpura which provide further support for Evans' hypothesis of immune mechanisms in idiopathic thrombocytopenia.

It has already been shown how Ackroyd's work (Ackroyd, 1949 a, b, c, & 1951, 1952, 1954) provided major advance in our understanding of the pathogenesis of thrombocytopenic purpura and it is desirable to consider his work in more detail. He demonstrated in vitro the probable mechanism of platelet destruction in the blood of patients recovering from thrombocytopenic purpura induced by the drug, 'Sedormid' - (allyl-isopropyl-acetyl carbamide). He found that plasma or

serum from such patients could, in the presence of both Sedormid and Complement, produce agglutination and lysis of both the patient's and normal platelets. Further, when the patient's platelet rich plasma was clotted in the presence of Sedormid, clot retraction was completely inhibited. No such reaction took place if normal serum or plasma was substituted for the patient's, or if the patient's serum and plasma was added to platelets in the absence of 'Sedormid'. He further found that Complement was fixed when platelet lysis was induced by the patient's serum in conjunction with Sedormid and that this reaction was specific (Ackroyd, 1951).

These observations suggested that Sedormid must unite with the patient's platelets and, acting as hapten*, confer antigenic properties upon them so that they will stimulate the formation of a platelet agglutinin or lysin in the patient's blood. These antibodies will react with the platelet drug complex and induced thrombocytopenia. On ceasing the administration of the drug, the antigenic complex disappears and the mechanism of platelet destruction can no longer operate (Ackroyd, 1955a).

Ackroyd's hypothesis has been confirmed in other cases where drugs have caused thrombocytopenic purpura.

Quinine	Grandjean 1948. Bolton & Young 1953
Quinidine	Bolton & Young 1953, Barkham & Tocantins 1954, Bigelow & Desforges

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* Hapten - a substance, itself non-antigenic, which can, when combined with a protein, confer specific antigenicity on that protein.

	1952, Larson 1953, Harrington 1954, Bolton & Dameshek 1956, Bolton 1956.
Sulphamezathine	Bolton & Young 1953
Antazoline	Ackroyd 1955b.
Dormisone	Stefanini 1954
Paraminosalicylic Acid	Wurzel and Maycock 1953.

Thrombocytopenic purpura may be caused by a large variety of drugs apart from those already quoted. A very full list of these has been given both by Ackroyd (1955a) and Stefanini & Dameshek (1955) and any further review here would be superfluous. Although, in many of these instances, in vitro sensitivity tests by Ackroyd's methods have not been performed it is probable that the underlying mechanism is the same in all.

Idiopathic thrombocytopenic purpura may develop suddenly in the newborn child and such cases have been the subject of several excellent reviews. Robson & Davidson (1950) reviewed the literature of this condition in relation to maternal purpura in pregnancy. In their series all the mothers of the affected neonates were or had been thrombocytopenic. A similar review by Epstein, Lozner, Cobbey & Davidson (1950) stressed the relatively high neonatal mortality and morbidity of this condition although transient and self limiting in the child.

Other cases of neonatal thrombocytopenia have been reported by Akerren & Rhinand (1950), Driere (1951), Stening (1953), Harrington et al (1953) Morris (1954), Sauer & Van Loghem (1955) and Peterson & Larson (1954), Vandenbroucke & Verstraete (1955), and Stefanini & Dameshek (1955). Many of these examples suggested that a humoral factor or antibody was transferred through the placenta from the thrombocytopenic mother to the child producing thrombocytopenia of variable duration and, in some cases, it was possible to determine that a specific antiplatelet factor did exist in the mother's and baby's serum. Both Harrington and Stefanini have stated that if no platelet antibody can be found in the blood of a thrombocytopenic mother, the child will not be affected (Stefanini, Mednicoff, & Plitman, 1954; Harrington, Minnich & Arimura, 1956). Harrington and co-workers (Harrington et al, 1953) together with Stening, (1953), Morris (1954), Sauer & Van Loghem, (1955), Stefanini, Mednicoff & Plitman (1954), Stefanini & Dameshek (1955) and Tullis (1956) have drawn attention to neonatal thrombocytopenia appearing in the children of a normal mother who had never shown any evidence of thrombocytopenia or purpura. This combination suggested that the mother must have become immunised to the baby's platelets due to their having an antigenic structure different from her own, a state of affairs analagous to the red cell Rhesus factor incompatibility in haemolytic disease of the newborn. In this group antibodies have been found in the

mother's blood which selectively agglutinate the father's platelets and those of certain other normals but not the mother's platelets (Harrington et al. 1953; Harrington 1954). Sauer & Van Loghem (1955) found that using Kissemeyer Nielsen's technique, that serum from normal mothers might contain some anti-platelet factor which would induce agglutination of tanned red cells coated with platelet protein made from the mother's own platelets. In view of the apparent non-specificity of the technique used in these experiments, no importance can be attached to this finding.

The analogy between neonatal thrombocytopenic purpura in children of normal mothers and haemolytic disease of the newborn, due to red cell group incompatibility, has supported other claims to have demonstrated a platelet group structure separate from that of red cells. (Harrington et al. 1953; Stefanini et al. 1953b).

If it be accepted that specific anti-platelet antibodies exist which are capable of destroying or damaging the platelets in certain cases of idiopathic thrombocytopenia and other related conditions, the majority of these must be agglutinins or lysins and more than one type must exist.

They may be defined as follows:

1. Allergic anti-platelet agglutinins or lysins found in cases of drug or toxic thrombocytopenic purpura.
2. Anti-platelet pan-agglutinins or lysins capable of agglutinating or lysing not only the patient's own platelets but also those derived from normal donors.

3. Anti-platelet auto-agglutinins or lysins which agglutinate or lyse the patient's platelets only and have no effect on suspensions of normal platelets.

4. Anti-platelet iso-agglutinins or lysins which agglutinate or lyse certain normal platelet suspensions but not those of the patient.

These terms will be used in the discussion to follow.

This review has summarised the evidence that has been produced in support of the Evans hypothesis. It is obvious that the major difficulty confronting workers in this field has been the establishment of suitable techniques for demonstrating anti-platelet factors in blood. Platelets will clump under the influence of certain blood coagulation and non-specific factors. Some workers have failed to recognise these pitfalls and their experimental findings must be discarded. Therefore, to confirm or refute these many claims, it was essential to make a personal survey of this problem using a technique which allowed for the presence of coagulation and non-specific platelet clumping factors.

Evans, Takahashi, Duane, Payne & Liu (1951)	5	Unspecified	5	Direct Platelet Agglutination Test Indicator: Normal platelet rich citrate plasma Test Serum heat treated 56°C for 30'
Harrington, Minnich, Hollingsworth & Moore (1951)	6	2 Acute ITP 1 Chronic ITP 3 Recurrent ITP	2 1 2	Transfusion technique Reduction of platelet count in normal recipients after transfusion with blood from cases of ITP.
Stefanini, Chatterjea & Adelson (1952)	20	Unspecified	3	Direct platelet agglutination "Various precautions were taken to avoid non specific agglutination"
Dausset, Delafontaine, Fleuriot (1952)	5	1 Acute ITP 1 Neonatal ITP 1 Chronic ITP 2 Secondary ITP	1 0 0 0	Direct Platelet Agglutination Test. Indicator: Normal platelet rich citrate plasma Test Serum (untreated)
Nolthenius, Hoorweg, & Van Loghem (1953)	9	Unspecified ITP	0	Director Platelet Agglutination Test (1) Indicator: Platelet suspension washed x2. Test Serum heat treated 56°C for 30'
	3	TP	3	(2) Test serum added to anti-human platelet rabbit serum potentiated the titre of the latter when tested against normal human platelets

Kissemeyer Nielsen (1953a)	Unspecified 3	Normal I.T.P. Rabbit antisera	"Few" "Some" 3	Indicator: Tanned red cell-platelet protein mixture. Serum: Patient's or rabbit's serum - untreated.

Harrington, Sprague, Minnich, Moore & Milvin & Dubach (1953)	35	22 Acute ITP 13 Chronic ITP	14 8	Direct Platelet Agglutination Test Indicator: Normal platelet rich sequestrene plasma. Test Patient's serum aged 24 hours + sequestrene + N/3 citric acid or platelet free plasma +N/3 citric acid
Stefanini, Dameshek, Chatterjea, Adelson & Mednicof (1953)	1	Chronic ITP	1	Direct Platelet Agglutination Test Indicator: Platelet rich sequestrene plasma Test: Patient's serum decalcified with ion exchange resin (IRC 50) or patient's platelet free plasma
Tullis (1953a)	18 26 9	Unspecified ITP Secondary TP Hypersplenism	9 1 6	Direct Platelet 'Lysis' Test Indicator: Platelets washed x3 in dextrose acetate saline Serum test Adsorbed with barium sulphate - carboxylated ion exchange resin (XE 128 Röhm & Haws Co.) Complement 1/32 normal human serum
Fluckiger, Hassig & Koller (1953)	5	Unspecified ITP	3	Direct Coombs test on patient's platelets

Moulinier & Mesnier (1954)	Unspecified	Acute ITP	2	Indicator: Cholesterol crystals + lipoid extract of platelets Test: Patient's sera untreated
Sauer & Van Loghem (1954)	34	24 Chronic ITP 3 Acute ITP 4 Unspecified ITP 3 Amegakaryocytic	11 0 2 0	Kissmeyer Neilsen Technique (Kissmeyer Neilsen 1953) Modified by the serum being heat inactivated and barium sulphate adsorbed prior to testing.
	47	Unspecified ITP	0	Direct Coombs Test on patient's platelets
Dausset (1954)	71	Acute ITP Chronic ITP	2 8	Direct Platelet Agglutination Test Indicator: Platelet rich sequestrene plasma Test Sera heat treated 56°C for 30' Agglutination accelerated by continuous agitation for 30'
Harrington (1954)	43 29	Acute ITP Chronic ITP	30 20	Direct Platelet Agglutination Test Indicator: Normal platelet rich plasma sensitised with acid pepsin & calcium chloride magnesium chloride mixture. Patient's serum heat treated 56°C for 30' (Enhance agglutination reaction by the addition of bovine-horse serum mixture - 'Conglutination reaction')

Stefanini (1955b)	51	Acute ITP	8	Direct Platelet Agglutination Test
	68	Chronic ITP	56	Indicator: Normal platelet rich plasma
	6	Drug TP	6	Test: Platelet free plasma or serum decalcified with ion exchange resin IRC 50
	2	Haem. Anaemia with ITP	0	
	1	Anaphylactoid purpura	1	
	132		30%	Direct Coombs Test (Patients' platelets)
Verstraete & Vandenbroucke (1955)	3	Unspecified ITP	2	Direct Platelet Agglutination Test
	2	(After splenectomy)	1	Indicator: Normal platelets washed x3
	10	Multiple transfusions	3	Patient's serum heat treated 56°C for 30' barium sulphate adsorbed + 1/10 sodium citrate
	2	Unspecified ITP	2	Kissmeyer Nielsen Technique
Malinvaud & Dausset (1955)	100	Unspecified ITP	12	Direct Platelet Agglutination Test
	41	Secondary ITP	2	Method as per Dausset 1954
	37	NTP	1	
	1320	Normals	20	

TABLE XXXII

Techniques used to detect anti-platelet factors in the blood obtained from cases of idiopathic thrombocytopenic purpura and related conditions. ITP = Idiopathic Thrombocytopenic Purpura. TP = Thrombocytopenic Purpura.

Originators	Total No. of cases tested	Classification and number of cases tested	Positive results.	Method
Moulinier (1955)	7 5 70	Unspecified ITP Secondary ITP Normals	7 5 1	Antihuman globulin Consumption Test (1) Normal washed or lyophilised platelets activated with papaine. (2) Activated platelets incubated with test serum (untreated) (3) Platelets washed x5 (4) Add to anti-human globulin (5) Titrate residual antihuman globulin against ORh+ cells sensitised with incomplete anti Rh antibody
Tullis (1956)	96 21 127 16 19	ITP unspecified Hypersplenism Secondary TP Neonatal TP N.TP	54 14 2 16 0	Direct Platelet Lysis Test Indicator: Normal platelets washed x3 in dextrose acetate saline Serum Patient's absorbed with barium sulphate-carboxylated ion exchange resin (XE128 R8hm & Hass Co) Complement 1/30 normal human serum
Weinrich (1956)	31 64 52	Unspecified ITP Secondary ITP Misc. NTP's	7 18 1	Direct Platelet Agglutination Test Indicator: Normal washed platelets. Test Serum: Heat treated 56°C for 30'
	31 64	Unspecified ITP Secondary ITP	0 0	Direct Coombs Test on Patients' Platelets

CHAPTER X

Platelet Agglutination Test

In the previous chapters the behaviour of platelets during the sequence of blood coagulation has been analysed in three separate experimental systems. The agents which produced viscous metamorphosis (VM) in these systems showed definite differences from one another, and although no one factor was common to all, it was possible to determine how the factors responsible for platelet VM could be destroyed or neutralised. Similarly, certain factors were found to produce non-specific platelet agglutination and these must be avoided if platelet agglutination is to be studied in vitro. Any platelet clumping that occurs in a system, in which both coagulation and non-specific factors have been excluded, must be a measure of yet another form of platelet agglutination.

In Chapter IX the claims of other workers to be able to demonstrate a platelet agglutination factor in the blood of certain cases with thrombocytopenia, have been described. Preliminary experiments by the author using some of these methods showed that their, apparently satisfactory, results could not be repeated. Therefore a platelet agglutination test was designed which excluded these coagulation and non-specific platelet agglutinating factors.

Platelet Agglutination Test. (The detailed technique is given in Appendix A, p.14).

Preparation of Platelet Suspensions

Platelets suspended in their own plasma have been shown to be more readily affected by any change in their environment and to be much more sensitive to residual amounts of coagulation factors. When separated from their plasma, and all traces of the latter removed by repeated washing, they became more resistant to environmental change. Moreover, well washed platelets were found to be resistant to serum viscous metamorphosing factors (Table VIII). Therefore it was decided to use washed normal platelet suspensions as the indicator in the platelet agglutination test. Achard & Aynaud (1908b) and Tocantins (1938) have claimed that the addition of many agents, foreign to the blood, will produce non-specific agglutination. They cited gelatin, peptone, egg albumen, egg lecithin, gum arabic, and colloidal arsenic as examples, and stressed that the one feature common to all was their colloidal nature. Therefore any materials used in the separation, washing, or resuspension of blood platelets had first to be examined in order that any, which might produce non-specific platelet agglutination, be excluded.

The Effect of Anticoagulant on Platelets

First, it was essential to decide the optimum anticoagulant for the preparation of adequate platelet suspensions. Sodium citrate, sodium oxalate, disodium versene, and heparin have all been used for the isolation of intact platelets from the blood. The criterion of the most suitable anticoagulant

was decided arbitrarily as that which would allow the platelets to stay suspended in their own plasma, without agglutination or lysis, for 18 hours at 37° (Experiment 31. Appendix B, p.22).

Only disodium versene (disodium ethylene diamine tetra-acetic acid) met with this criterion (Table XXXIII). This powerful calcium-binding salt has strong affinity for divalent ions and has proved to be the best anticoagulant for the preservation of platelets (Proescher 1951). Further, it is widely cited as the coagulant of choice for the preparation of platelet concentrates for intravenous transfusions (Dillard, Brecher & Cronkite 1951).

The Separation of Platelets from the Plasma

The platelets were separated from their plasma by high speed centrifugation and resuspended in various isotonic salt solutions. This process was repeated three times in order to remove all traces of plasma from the platelets. Various isotonic solutions were assayed for their suitability as platelet washing fluids (Experiment 32. Appendix B, p.22). After the platelets had been washed three times, and resuspended in the same fluid, they were examined for the presence or absence of platelet agglutination (Table XXXIV).

The best solution was found to be dextrose-acetate-saline (DAS), as recommended by Tullis (1953a). Not only did this solution provide an agglutinate-free suspension of platelets but the morphology of the surviving platelets was better than

TABLE XXXIII Experiment 31 Appendix B, p22

The effect of varying anticoagulants on the stability of platelet suspensions.

Samples of platelet rich plasma collected in varying anticoagulant mixtures were incubated for 18 hours at 37°C in siliconed tubes. Any alterations in platelet morphology were then recorded.

Anticoagulant	Concentration of Anticoagulant	Platelet Agglutination	Platelet Lysis
Sodium Citrate	3.8% 1 part to 9 parts blood	+	-
Sodium Oxalate	1.34% 1 part to 9 parts blood	-	+
Disodium Versene	4.5% 1 part to 33 parts blood	-	-
Heparin	10 u/ml	++	-

TABLE XXXIV Experiment 32 Appendix B, p. 22

The optimum medium for washing platelets free from plasma.

Platelets were separated from platelet rich 'Versene' plasma (Experiment 31) and washed three times in the following isotonic solutions, and finally resuspended in the same solution. Any alteration in platelet morphology was recorded.

Isotonic Salt Solution	Platelet Agglutination	Platelet Lysis
0.85% Saline	±	+
0.85% Citrate Saline	+	-
5% Glucose in 0.85% Saline	±	-
5% Glucose	±	-
(Dextrose) Glucose-acetate-saline (Tullis 1953)	-	-
1% Disodium Versene in 0.85% Saline	-	-
(Dextrose) Glucose Acetate Saline buffered to pH 7.3 with Glyoxaline Buffer	±	-

+ = denotes definite agglutination or lysis

± = denotes occasional platelet clumps

- = unaltered platelet morphology

when other solutions were employed. Further, the platelets resuspended easily in this medium and thus were washed more efficiently.

The pH of these isotonic solutions was found to be an important factor in the prevention of non-specific agglutination. Whenever the pH of these washing solutions fell below 6.0, non-specific agglutination took place immediately. This reaction was even more intense when the pH was less than 5.5. Raising the pH above neutrality to pH 8.5 did not alter the platelets in any way.

These observations agreed with those of Tocantins (1938) and Harrington (1954) who both reported that lowering the pH would produce platelet agglutination. The latter considered that if the pH fell below 6.6, non-specific agglutination might occur.

In an attempt to nullify this factor, isotonic glyoxaline buffer was added to DAS solution in the proportion of one part of buffer to nine parts DAS. This resulted in a solution of pH 7.1 which should not have induced any platelet changes. Unfortunately, when used to wash platelets, this buffer - DAS solution - was found to produce partial platelet agglutination. This impasse was circumvented by using freshly made distilled water to prepare each batch of DAS. Small batches of DAS were made up and used within one week, the pH being checked prior to each experiment. With these precautions, the pH was maintained above 6.8.

Media for the Suspension of Platelets

The platelets, having been separated from their plasma and washed, had to be suspended in some suitable medium before addition to test serum for a fixed period of incubation. It was essential that the suspending medium did not promote non-specific agglutination during this period of incubation and so various suspending media were examined (Experiment 33. Appendix B, p.23). The only suitable media were DAS, 2% gelatine in DAS, and 1% disodium versene in saline (Table XXXV). The 2% concentration of gelatine in DAS conferred stability on the platelet suspensions whereas 5% gelatine provoked definite agglutination.

Red Cells and Platelet Agglutination

During the course of these experiments, it was observed that, if platelet rich plasma, containing an appreciable number of red cells in suspension, was centrifuged at high speed, the platelets agglutinated or, in combination with the red cells, formed mixed agglutinates. These agglutinates were resistant to resuspension even after repeated washing. That the red cells alone were implicated in this phenomenon was shown by selectively packing platelets and white cells together in plasma. Neither the platelets or white blood cells showed any evidence of agglutination and could be resuspended easily.

To define the conditions under which this red cell-platelet agglutination phenomenon occurred, various experimental mixtures were examined.

TABLE XXXV Experiment 33 Appendix B, p.23

A suitable medium for preserving platelets in suspension.

Normal human platelets were separated from versene plasma and washed three times in dextrose-acetate-saline. Then they were resuspended in the following and platelet morphology observed after the suspension had stood in siliconed tubes at varying temperatures for 3 hours.

Suspending Media (Concentration)	Presence or Absence of Platelet Agglutination		
	Temperature		
	37°C	Room Temp.	4°C
Saline 0.85%	+	+	-
Citrate Saline 0.85%	+	+	-
1% Disodium Versene in 0.85% Saline	-	-	-
Glucose 5%	±	-	-
5% Glucose in 0.85% Saline	+	±	-
Dextrose Acetate Saline (DAS)	-	-	-
2% Gelatine in DAS	-	-	0
5% Gelatine	+	±	0
20% Bovine Albumin (Armour)	+	+	+
Human Albumin (Lister Inst.)	-	±	-
Egg Albumin	++	++	+
Human Fibrinogen	±	-	-

0 = No observation

++ = Strong platelet agglutination

+ = Definite platelet agglutination

± = Occasional platelet clumps

- = No agglutination

Platelets and red cells obtained by differential centrifugation from the same blood were washed separately in DAS and, after each washing, the equal volumes of washed platelets red cells were mixed and forced together by high speed centrifugation. The presence or absence of platelet or mixed agglutination was then observed by phase contrast microscopy (Experiment 34. Appendix B, p. 24)

Only when platelets and red cells are packed together in plasma did this platelet or mixed red cell platelet agglutination take place. Washing either series of cells inhibited the phenomenon (Table XXXVI). Therefore some factor must be removed easily from the red cell or platelet surface by washing.

The individual supernatants from the first washing of both platelets and red cells were re-added to washed platelet-red cell mixtures in an attempt to restore the agglutination phenomenon. Neither separately nor in combination could these saline eluates of these cells restore their ability to agglutinate.

In a further attempt to unravel this problem, washed red cells were added to platelet rich plasma after exposure to trypsin or incomplete immune anti-Rhesus antibody of known potency in an attempt to restore the ability of these washed red cells to promote this phenomenon (Experiment 35. Appendix B, p. 25).

The effect of washing on the platelet red cell agglutination phenomenon.

Before and after washing a mixture of platelets and red cells were packed together by centrifugation and then resuspended. The presence or absence of platelet or mixed agglutination was recorded.

Normal Platelets	Presence or Absence of Platelet or mixed Platelet-Red Cell Agglutination			
	10% Red Blood Cells			
	In Plasma	Wash 1	Wash 2	Wash 3
In Plasma	++	-	-	-
Wash 1	-	-	-	-
Wash 2	-	-	-	-
Wash 3	-	-	-	-

When washed reds were coated with an incomplete (non-agglutinating) immune antibody the phenomenon of mixed red cell-platelet or platelet agglutination could be observed (Table XXXVII). Incomplete anti-red cell immune antibodies do coat red cells, carrying the appropriate antigen, with a layer of globulin and it is possible that this protein could be the essential link between the platelets and red cells. This non-specific adherence of platelets and white cells to red cells coated with immune antibody has been observed by Swisher (1956) who considered that this mechanism might be of importance in incompatible blood transfusions. Thus one cannot be certain that the coating of red cells with incomplete antibody did restore the 'status quo ante' and that this phenomenon is not entirely separate from the original observation of platelet red cell agglutination in high spun plasma.

This "platelet-red cell-agglutination" phenomenon may be the same as that described by Pennel (1943) who observed "Platelet-red cell conjugation" when whole blood clotted but did not observe the same phenomenon in citrated blood; the agglutination observed in the present series was, however, much more marked than that illustrated by Pennel. It is known that normal red blood cells carry a layer of globulin on their surface (Stratton & Jones 1955) and it is not unreasonable to propose that, when platelets are forced into contact with this layer, some unknown alteration in their surface properties may allow them to adhere to the red cell or to one another.

The effect of trypsin and incomplete antibodies on platelet red cell agglutination.

0.5 ml of a 10% suspension of modified red cells were added to 0.5 ml platelet-rich plasma or 0.5 ml once washed platelets in D.A.S. Red Cells and platelets were packed together by high speed centrifugation, resuspended, and the presence of platelet or mixed red cell-platelet agglutination recorded.

Modified Red Cells	Presence or Absence of Platelet Agglutination	
	Platelet Rich Plasma	Washed Platelet Suspension
Trypsinised Red Cells	-	-
Red cells sensitised with incomplete immune antibody	++	-

Whatever the explanation, the practical importance of this observation is clear. If non-specific agglutination of platelet suspensions is to be avoided, all but trace amounts of red cells must be removed from the platelet rich plasma before platelets can be separated successfully from plasma by high speed centrifugation.

Bacteria and Platelet Agglutination

Aynaud (1911b) noted that when certain bacteria were added to platelet rich plasma, platelet agglutination took place. In the present experiments, accidental bacterial contamination of stored platelet suspensions or test sera resulted invariably in marked non-specific platelet clumping. Therefore all anticoagulant solutions used in these experiments and platelet washing solutions were sterilised prior to use and all sera were separated and stored in sterile universal containers at -15°C.

Preparation of Test Sera

Serum, unaltered by anticoagulants, is, in the experience of the majority of serologists, a far more reliable agent for demonstrating immune reactions than plasma, and has been used, with certain exceptions, in all the experiments to be described. As unaltered fresh serum contains a factor capable of inducing VM in washed platelets (Chapter V), it was necessary to destroy this property of serum. The addition of decalcifying anticoagulants was the simplest method available (Table XIII), but this would not only dilute the serum but restore it to a state identical to that existing

in plasma. Therefore, to exclude all residual coagulation factors, the test sera were treated as follows:

(a) the serum was 'aged' by standing at 37°C in contact with the original clot;

(b) the serum was heat treated at 56°C for 1 hour. This was found to be essential for complete destruction of the serum VM factor (Table XIII). This treatment destroyed Complement completely and therefore platelet lysis was not expected to occur in the test used in this series;

(c) finally, the serum was adsorbed by barium sulphate powder.

Any one of these methods was, by itself, capable of destroying the ability of the serum to induce VM in washed platelets, but was not able to destroy the ability of serum to produce mild agglutination after prolonged incubation with platelets.

1	0	$R_1^+R_1^-$	MN	−	−	+	− +	+	+	−	−
2	0	$\overset{-}{xr}$	MN	−	−	−	−	− −			−
3	0	R_1^+r	MN	+					+		−
4	0	$\overset{-}{xr}$									
5	A	$R_1^+R_1^-$	MN	+	−	+	− +	+			−
6	0	$R_1^+R_1^-$	M	+	−	+	− +	+	+	−	−
7	0	$\overset{-}{xr}$	M	+	−	+	− +	−	+	−	−
8	B	R_1^+r	M	+	−				+		−
9	A	R_1^+r	MN	+			− +	+			−
10	A	$R_1^+R_2^-$	MN	+	−	+	− +	+ +			

11	O	$\overset{-}{rr}$	M	+	-	+	-	+	-	+	+	-
12	A	+										
13	O	$\overset{-}{rr}$	MN	+			-	+	-	-	+	-
14	A	$\overset{+}{R_1}r$	MN	-					+			
15	O	$\overset{+}{R_1}R_1$	N	-	-	-	-	+	+	+	+	-
16	A	-										
17	A	$\overset{+}{R_2}r$	MN		-		+		-			-
18	A	$\overset{+}{R_1}R_1$	M	-	-		-	+	-			-
19	A	$\overset{+}{R_1}R_1$	N	-			-	+	+			+
20	A	$\overset{+}{R_1}r$		-	-		-	+	-	+	-	-

21	A	+								
22	O	$\overset{-}{rr}$	N	+	-	+	+	+		-
23	B	+								
24	A	$\overset{+}{R_1}r$	MN	+			- -			
25	O	$\overset{+}{R_2}r$	MN	+	-	+	- +	-	- +	-
26	A	+								
27	B	+								
28	A	+								
29	O	$\overset{+}{R_1}r$	M	-	-	-	- +	+	- +	-
30	A	$\overset{+}{R_1}r$	M	+	-		+ -	+		

Table XXXVIII

Blood Groups of Normal Platelet and Serum Donors

Identification Number of Donor	ABO Group	Rhesus Group & Genotype	MN Group	S Group	Lutheran Group	P Group	Lewis Group Le a	Lewis Group Le b	Duffy Group Fy a	Duffy Group Fy b	Kidd Group JK a	Kidd Group JK b	Kell Group K
31	O	$\overset{+}{R_2}r$	MN	-	-	+	-	+	+				-
32	A	+											
33	O	$\overset{+}{R_1}r$	MN	+	-	+	-		+				-
34	O	$\overset{+}{R_2}r$	MN	-	-	-	-		+				-
35	A	$\overset{+}{R}or$	MN	-	-	+	-		-		-	+	-
36	O	$\overset{-}{rr}$	M	+	-	+	-	+	-		+	+	-
37	A	$\overset{+}{R_1}R_2$	N	-	-	+	+	-	+				-
38	A	$\overset{+}{R_1}R_1$	MN	+	-		-	+	+		+	-	
39	B	$\overset{+}{R_1}R_2$		+	-		-	+	+				
40	O	$\overset{-}{rr}$	MN	-	-	-	-	-	+		+	+	-

CHAPTER XI

Platelet Agglutination in Normals

Before it could be determined that any acquired immune platelet agglutinating factors existed in the sera from cases of idiopathic thrombocytopenic purpura and other related syndromes, it was important to determine whether any similar factors could be found in sera prepared from normal blood. Therefore Platelet Agglutination Tests were performed using blood from forty normal subjects.

Normal healthy male and female volunteers were selected at random from the medical and technical staffs of the United Oxford Hospitals and tested in groups of ten. The red cell blood group structure of all these donors was determined (Table XXXVIII). None of these normals had received any blood transfusions nor had any of the females been pregnant. Thus it could be assumed that none of the sera prepared from these normals contained any immune auto- or iso-agglutinins. Washed platelet suspensions were prepared from 10 normals and added to treated sera from the same 10 persons so that every platelet suspension was tested against every serum, involving 100 tests in all (Experiment 36. Appendix B, p. 26). The results of the first one hundred tests are recorded in Fig. 21.

This experiment was repeated at random 10 times until 1000 individual platelet agglutination tests had been performed (Experiment 36. Appendix B, p. 26), using the blood from 40 separate donors. Some of the

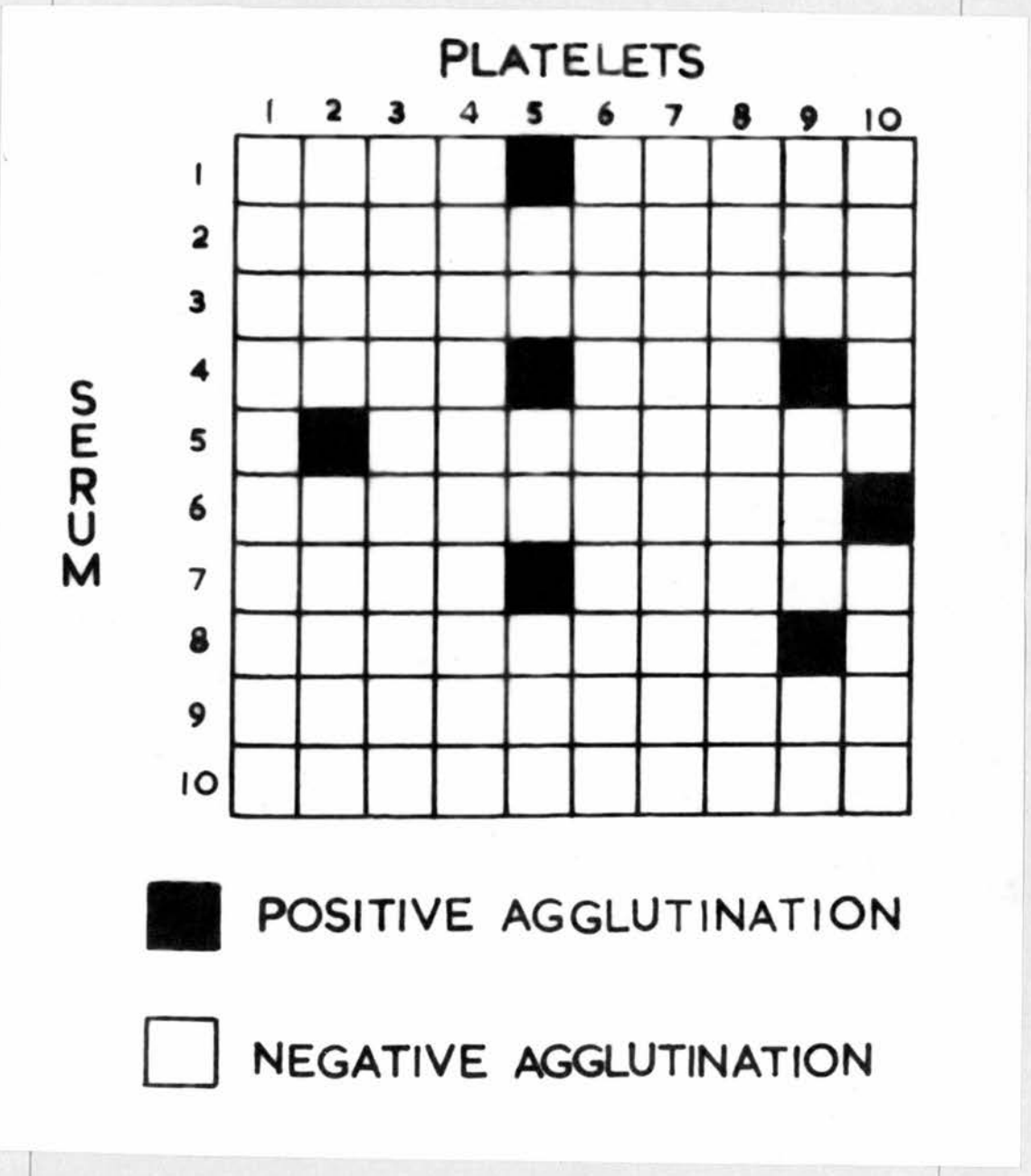


Figure 21

The results of 100 platelet agglutination tests performed by mixing 10 normal sera with 10 normal platelet suspensions in all possible combinations.

platelet-serum combinations were tested by necessity several times and not all the 1600 possible combinations were examined. A summary of the ten experiments is given in Fig. 22.

A surprisingly high incidence of positive results was found in this survey. In 1000 tests, 72 positive agglutination tests were found and it was apparent that certain platelet suspensions were more readily agglutinated by normal sera than others. This suggested platelets might be divisible into groups akin to red blood cell groups or that they had a separate group structure of their own.

Platelet Groups

That platelet groups might exist had been suggested by Toda (1923), Harrington et al (1953), Stefanini, Plitman, Dameshek, Chatterjea & Mednicoff (1953). These workers found that there was no evidence of any inter-relationship between possible platelet groups and the ABO system of red blood cell groups. However, Gurevitch & Nelken (1954, 1955a & b), Moureau & André (1954), and Dausset et al (1954) claimed that platelets did in fact carry the same ABO antigen as their corresponding red cells.

Platelets and the ABO Blood Group System

It was decided to analyse the results illustrated in Fig. 22 to see if there was any evidence of A & B red cell antigens on the platelets, or alternatively whether any other platelet group pattern could be determined, and so determine which of the above claims was correct.

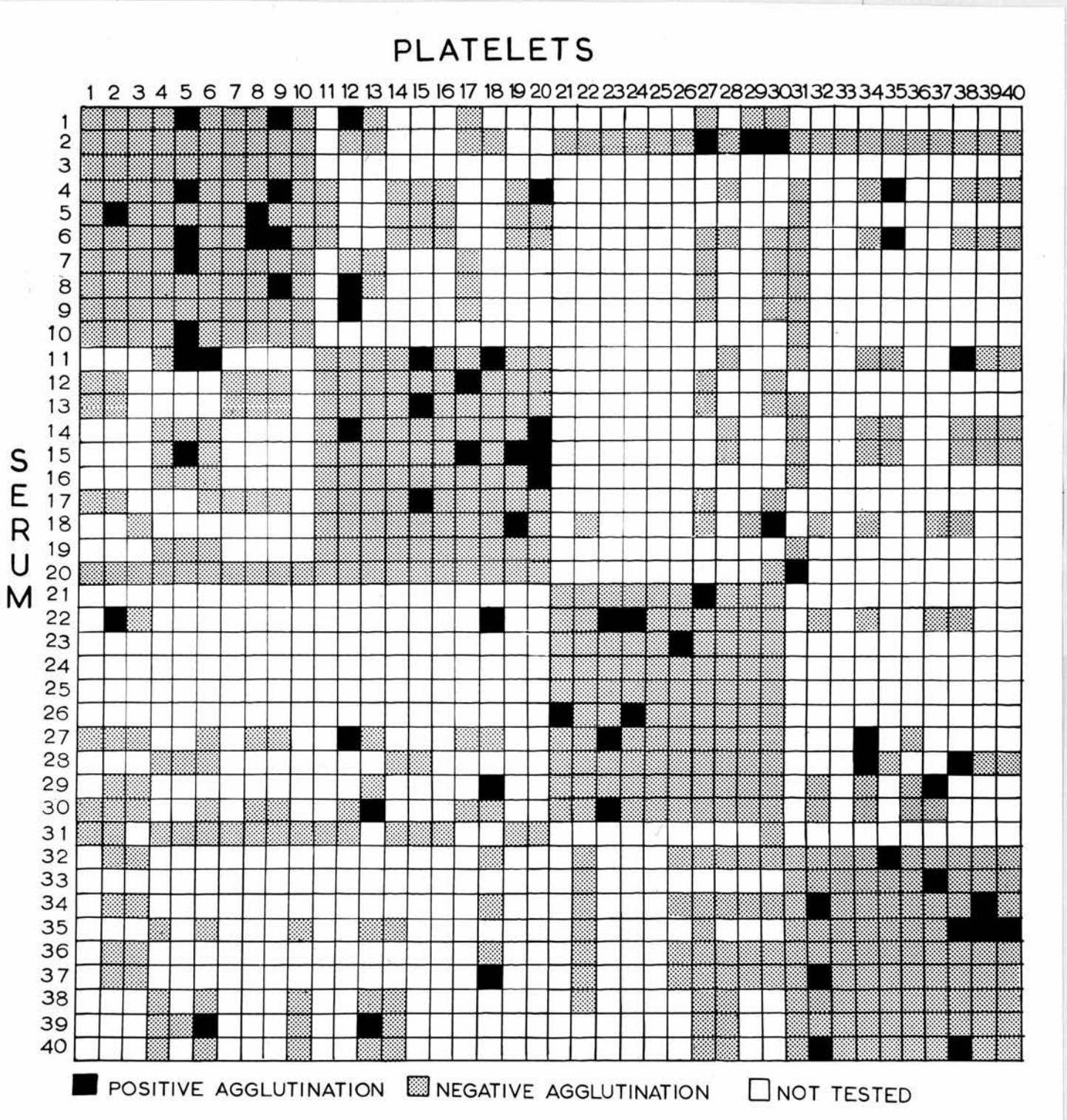


Figure 22

The results of 1000 platelet agglutination tests performed on samples of normal blood. This figure incorporates the results of ten experiments involving random mixtures of normal sera and suspensions of normal platelets prepared from blood derived from 40 normal donors.

Table XXXVIII demonstrates the distribution of ABO and Rhesus groups in the 40 normal donors used in these tests. The remaining red cell group structure was known only in 29 individuals, but this number was sufficient to give samples of sufficient numbers for reliable analysis.

The 72 positive results were analysed into groups according to the known group structure of the red cells of the donor from which the serum and platelets were derived (Table XXXIX). From this analysis the numbers of positive results appeared to vary according to the ABO and Rhesus groups of the blood from which the platelets were derived. Therefore, it appeared to be possible that the platelets did carry the ABO and Rhesus antigens. If this were so, it would be expected that all the platelets which had been cross-matched with sera of incompatible ABO groups should be agglutinated. The results were therefore re-analysed in terms of compatible and incompatible ABO groups (Table XL).

Far from all the incompatible combinations being agglutinated only 14.19% gave positive results. This figure was higher, however, than that obtained from compatible groups. Statistical analysis of these differences (Appendix D, p. 2) showed that P equalled less than 0.01. Therefore this difference was significant and unlikely to be due to chance.

But, although these results were suggestive, the hypothesis that platelets contained the ABO group antigens was

Table XXXIX

Analysis of positive platelet agglutination Tests (Fig 22) and other in terms of the ABO/groups of platelet donor blood and serum donor blood. (See also Table XXXVIII)

Red Blood Cell Group of Platelet Donor	Red Blood Cell Group of Serum Donor	Total No. of Combinations Tested	Total No. of Positive Agglutination	Percentage of Positive Agglutination Tests
O	O	233	5	2.14
O	A	193	5	2.59
O	B	44	2	4.54
A	O	193	30	15.54
A	A	214	16	7.47
A	B	33	5	15.15
B	O	44	4	9.09
B	A	33	4	12.12
B	B	13	1	7.69
Rh+	All Groups	720	67	9.30
Rh-	"	280	5	1.78
Rh+	Compatible ABO Groups	312	20	6.4
Rh-	"	137	2	1.46
MN	All Groups	390	31	7.94
M	"	240	14	5.83
N	"	100	7	7
S+	All Groups	490	33	6.73
S-	"	380	24	6.32
P+	All Groups	310	16	5.16
P-	"	160	9	5.62
Fy (a+)	All Groups	520	38	7.3
Fy (a-)	"	210	12	5.71
Le (a+ b-)	All Groups	80	4	5
(a- b+)	"	480	39	8.25
(a- b-)	"	140	8	5.71
JK (a+ b-)	All Groups	130	10	7.69
(a- b+)	"	90	7	7.77
(a+ b+)	"	120	4	3.33

Kell) Insufficient variation of these groups among the
Lutheran) donors rendered any analysis impossible.
Fy(b) Only one of the donors had had this group tested.

Table XL

Analysis of Positive Platelet Agglutination Tests (Fig.[22]) in terms of ABO compatibility between platelets and sera. (See also Table XXXIX)

Group Compatibility	Red Blood Cell Group of Platelet Donor	Red Blood Cell Group of Serum Donor	Total Number Tested	Total Number Positive	Percentage of Positive Agglutination Tests
Compatible Mixtures	O	O	697	29	4.16
	O	A			
	O	B			
	A	A			
	B	B			
Incompatible Mixtures	A	O	303	43	14.19
	A	B			
	B	O			
	B	A			

still not proven.

Determination of ABO Platelet Groups by Specific Antisera

In a further attempt to confirm or refute this hypothesis, washed suspensions of platelets, derived from blood of known ABO group, were specifically tested against sera containing anti A and B iso-agglutinins of known potency (Experiment 37, Appendix B, p.27).

These specific agglutinins agglutinated only some of the platelets which possibly contained A and B antigens (Table XLI). They did not agglutinate platelets from Group O or from A or B group blood derived from persons who were non-secretors of A and B substance in their saliva. (These were selected from those normals with red cell sub group $Le^{a}+$ who are known to be non-secretors of blood group substance (Race & Sanger 1954)). Further, only high titre sera and not routine blood grouping sera gave reliable results.

These observations suggested that either the amount or concentration of A and B antigen on the platelets was considerably less than their corresponding red cells, or if the platelets from non-secretors of A and B substance could not be agglutinated, the A and B antigenicity of platelets might be due to A and B substance adsorbed by the platelets from the circulating blood.

If this latter hypothesis was correct, it might be possible to dislodge this adsorbed material by repeated washing of the platelets (Experiment 38. Appendix B, p.28).

Table XLI Experiment 37 (Appendix B, p.27)

To determine the presence or absence of A & B antigens in platelets.

Washed platelet suspensions were prepared from donors of known red cell group and added to an equal volume of Anti A and Anti B grouping sera. After incubation the presence or absence of platelet agglutination was determined.

Red Cell Group of Platelet Donor	Platelet Agglutination			
	High Titre Sera		Low Titre Sera	
	Anti A	Anti B	Anti A	Anti B
O	-	-	-	-
O	-	-	-	-
O	-	-	-	-
O	-	-	-	-
O	-	-	-	-
A_1 Secretor	+	-	+	-
A_1 Secretor	+	-	-	-
A_1 Secretor	+	-	+	-
A_2 Secretor	±	-	-	-
A_1 Non Secretor	-	-	-	-
A_1 Non Secretor	-	-	-	-
B Secretor	-	+	-	-
B Secretor	-	+	-	-
B Secretor	-	+	-	-
B Non Secretor	-	-	-	-
B Secretor	-	+	-	+
AB	+	+	-	+

High Titre Serum — Anti A substance, Anti B substance } Rabbit sera

Titre (Anti A v. Group A red cells) 1/5120 (v. Group A platelets) 1/64

(Anti B v. Group B red cells) 1/1560 (v. Group B platelets) 1/32

Low Titre Serum — National Blood Transfusion serum (routine issue)

Titre (Anti A v. Group A red cells) 1/256 (v. Group A platelets) 1/2 or nil.

(Anti B v. Group B red cells) 1/128 (v. Group B platelets) 1/1 or nil.

After 10 washes, however, platelets from Group A and B secretors could still be agglutinated by their respective high titre antisera (Table XLI. A).

Mixed Red Cell Platelet Agglutination Test

This problem was eventually decided by utilising the method described by Coombs & Bedford (1955) for determining the antigenicity of platelets. This they called the 'mixed red-cell-platelet agglutination test'.

They evolved this test after they had also decided that the demonstration of the A and B antigens on platelets was not so straight forward as other workers had suggested. They claimed that if platelets and red cells shared the same antigenic structure they would show a definite pattern of mixed agglutination in the presence of the appropriate antibody. If they did not share the antigen, either the red cells or the platelets might agglutinate separately, but never together. The mixed red cell platelet agglutination they illustrated as demonstrating a specific appearance peculiar to their test.

The test was divided into two stages. The first involved preliminary incubation of the test platelet suspension with specific antisera. The platelets were then separated from the antisera by washing and red cells of known antigenicity were now added to the platelets and any potential mixed agglutination provoked by centrifugation of the mixture for a short period (Experiment 39. Appendix B, p.28).

Table XLIA Experiment 38 (Appendix B, p. 28)

To determine whether platelets carry adsorbed A or B substance on their surface.

Platelet suspensions were prepared from blood of known ABO blood group and repeatedly washed. After each washing the platelet suspensions were exposed to an equal volume of high titre anti A & B serum and the presence or absence of platelet agglutination determined.

Number of Washings	Antiserum	Platelet Agglutination		
		Red Cell Group of Platelet Donor		
		A	B	O
1	Anti A	+	−	−
	Anti B	−	+	−
2	Anti A	+	−	−
	Anti B	−	+	−
3	Anti A	+	−	−
	Anti B	−	+	−
4	Anti A	+	−	−
	Anti B	−	+	−
5	Anti A	+	−	−
	Anti B	−	+	−
6	Anti A	+	−	−
	Anti B	−	+	−
7	Anti A	+	−	−
	Anti B	−	+	−
8	Anti A	+	−	−
	Anti B	−	+	−
9	Anti A	+	−	−
	Anti B	−	+	−
10	Anti A	+	−	−
	Anti B	−	+	−

Their test was applied to confirm or refute the hypothesis that Group A or B non secretors did not carry A and B antigen on their platelets.

The results obtained (Table XLII) proved conclusively that A and B antigens were present on the red cells irrespective of whether the donor was a secretor or non secretor of blood group substance.

In conclusion, human platelets were found to share the ABO blood group system with their corresponding red cells.

It is difficult to reconcile this conclusion with the small proportion of positive ABO incompatible cross agglutination tests. Yet this finding is not surprising in view of the inability of specific anti A and B sera to agglutinate all platelet suspensions containing the opposite antigen.

Platelet Groups other than the ABO System

Further study of Table XXXIX showed two unexpected results.

Platelets derived from blood of group A and B cross matched with serum derived from group A or B blood respectively, and therefore compatible with the AB antigens, were agglutinated more frequently than platelets derived from Group O blood.

Furthermore, platelets derived from blood whose red cells were Rhesus positive agglutinated more readily than those derived from Rhesus negative donors. These differences

Table XLII Experiment 39 (Appendix B, p. 28)

Mixed Red Cell-Platelet Agglutination Test (Coombs & Bedford, 1955) to confirm the presence of A or B antigens in platelets.

Washed platelet suspensions (x 3) prepared from donors of known blood group were incubated with Anti A or Anti B sera for 1 hour at 37°C. The platelets were then separated from the sera, washed x 2, and added to an equal volume of group O, A or B red cells and the presence of mixed red cell-platelet agglutination determined.

Red Cell Group of Platelet Donor	Antiserum	Mixed Red-Cell Platelet Agglutination		
		Red Cell Group		
		O	A	B
O	Anti A	-	-	-
	Anti B	-	-	-
A (Secretor)	Anti A	-	++	-
	Anti B	-	-	-
A (Non Secretor)	Anti A	-	++	-
	Anti B	-	-	-
B (Secretor)	Anti A	-	-	-
	Anti B	-	-	+
B (Non Secretor)	Anti A	-	-	-
	Anti B	-	-	++
AB	Anti A	-	+	-
	Anti B	-	-	+

were significant, as with one degree of freedom, the values for P were <0.01 and <0.05 respectively (Appendix D, p. 3).

Platelets and Forssman Antigen

The fact that Group A and B platelets are more readily agglutinated than Group O platelets suggested that the former might carry an antigen, unrelated to Group O blood, which reacted with an agglutinating antibody present in normal serum. The Forssman antigen has been found to be associated with the A antigen (Kabat 1956) and, as some normal sera do contain Forssman antibody, experiments were performed to determine whether human platelets carried the Forssman antigen. It had been previously claimed by Cruz & Faro (1954) and Nicola, Rosti & Zangaglia (1956) that, while cat, rabbit, dog, goat, sheep, guinea pig and horse platelets carry the Forssman antigen, human platelets did not do so. Their experiments, however, did not define any blood group separation of human platelets and therefore a further investigation was indicated. A modification of the mixed red cell-platelet agglutination technique described by Coombs et al was used.

Potent Forssman antibody was prepared by immunising a rabbit with successive intravenous and intra-peritoneal injections of sheep red cells. (Experiment 40. Appendix B, p.30).

The anti-serum, both before and after adsorption with guinea pig kidney (to selectively adsorb the Forssman antibody (Wilson & Miles 1946)), was tested for its ability to promote

mixed red cell-platelet agglutination by the method of Coombs & Bedford (1955). Using sheep red cells as the test antigen no mixed red cell-platelet agglutination was obtained with anti-Forssman sera and therefore neither Group A nor Group B platelets appeared to carry the Forssman antigens (Table XLIII).

Platelets and the Rhesus Antigen

The difference in the number of positive platelet agglutination tests obtained, when suspensions of platelets were prepared from bloods whose Rhesus groups were positive or negative (measured by the presence or absence of the D antigen) was significant. The value for P was less than 0.05 (Appendix D, p. 3). Such a value is usually considered to be significant in biological research, but Race & Sanger (1954) require a value for P of less than 0.01 before they consider any results obtained in a survey of the incidence of blood groups, to be worthy of further investigation. Further, as none of the platelet donors used in these tests had received blood transfusions nor had been pregnant, the normal sera should not have contained immune Rhesus iso-agglutinins. As anti-Rhesus antibodies are never found as naturally occurring agglutinins, it is unlikely that these results were due to the presence of the Rhesus antigen on the platelets.

However, in order to confirm this view, the mixed red cell-platelet agglutination technique of Coombs et al (1955) was adapted to determine whether the Rhesus antigen detected on the platelets (Experiment 41. Appendix B, p. 31).

Table XLIII Experiment 40 (Appendix B, p.40)

Mixed Red Cell-Platelet Agglutination Test to detect the presence of Forssman antigen in platelets

Washed platelet suspensions (x 3) prepared from donors of known ABO blood groups were incubated with rabbit serum containing Forssman antibody (1) rabbit serum from which Forssman antibody had been removed by specific absorption with guinea pig kidney (2) for 1 hour at 37°C. The platelets were then separated from the sera, washed and added to suspensions of sheep red cells (Forssman antigen) and human red cells (no Forssman antigen) and the presence or absence of mixed red cell-platelet agglutination determined.

Red Cell Group of Platelet Donor	Rabbit Antiserum	Mixed Red Cell Platelet Agglutination	
		Sheep Red Cells	Human Red Cells
O	1	-	-
	2	-	-
O	1	-	-
	2	-	-
A	1	-	-
	2	-	-
A	1	-	-
	2	-	-
A	1	-	-
	2	-	-
B	1	-	-
	2	-	-
B	1	-	-
	2	-	-
B	1	-	-
	2	-	-

As no positive results were observed (Table XLIV), it was reasonable to assume that the Rhesus antigen D was not carried on human blood platelets. No studies were made to determine the presence of the other sub groups of the Rhesus factor, i.e. D.E., c.d.e. as these were not considered to be relevant to the present investigation. Ashurst, Bedford & Coombs (1956) have also found that the Rhesus D is not present on platelets and in addition showed that the antigens C.E. and c were not carried by platelets.

Platelets and Other Red Cell Blood Groups

In Table XXXIX the analysis of positive agglutination tests applied to other blood group antigens, i.e. M.N.P.S., Kell, Duffy and Lewis, did not reveal any other significant differences in the percentage of positive results obtained. These results did not exclude the presence of any of these antigens on the platelets but, as this survey had been made to determine the incidence of positive platelet agglutination tests in normal blood, their influence on these tests was obviously negligible and any further investigation was not considered to be necessary.

It has recently been claimed that the platelets do carry antigens other than those of the ABO group (Ashurst et al 1956). They found evidence which showed that the platelets carried the M and N and Tj a antigens in parallel with the red cells of their donor blood. This is an important advance in this field of research and further experiments must be performed

TABLE XLIV Experiment 41 Appendix B, p.31.

Human platelets and the Rhesus antigen.

Washed platelet suspensions prepared from Group O Rhesus + and Rhesus -ve donors were incubated with specific anti-Rhesus serum (incomplete anti-D). The platelets were separated and added to an equal volume of sensitised Group O Rehsus + and Rhesus -ve red blood cells together with one volume of anti-human globulin rabbit serum.

Red cell group of platelet donor	Rhesus Anti-serum	Mixed red cell platelet agglutination	
		Sensitised human red blood cells	
		ORh+	ORh-
O Rh+	Anti D	-	-
O Rh+	Anti D	-	-
O Rh-	Anti D	-	-
O Rh-	Anti D	-	-

to determine whether these findings have any practical significance in terms of platelet agglutination tests and platelet transfusions.

Platelets and Individual Platelet Groups

These experiments have shown that apart from the ABO antigens, no other known red cell blood group appears to influence platelet agglutination. (No analysis has been possible in terms of the antigen Tja as this group had not been determined on the red cells of any of the 40 donors used in these tests). If the positive results, due to ABO incompatibility between the platelets and serum, were excluded, a significant number of positive platelet agglutination tests still remained (Table XL). These might be due to the presence of a separate platelet group system and the presence of naturally occurring platelet iso-agglutinins in normal serum. Alternatively, if the latter assumption was not made, these could represent false positive results due to inadequacies of the technique.

On the assumption that platelet groups might exist, the positive results, unexplainable in terms of ABO incompatibility, were subdivided into the following categories:

(1) Platelet suspensions which were not agglutinated by any serum and whose corresponding serum did not agglutinate any other platelet suspensions.

(2) Platelet suspensions which were never agglutinated but whose corresponding serum did agglutinate other platelet suspensions.

(3) Platelet suspensions which were agglutinated but whose corresponding serum did not agglutinate other platelet suspensions.

(4) Platelet suspensions which were agglutinated and whose corresponding serum agglutinated other platelet suspensions.

These subdivisions were made from data obtained from Fig. 22, which is summarised in Table XLV.

In the fourth group listed above it was obvious that if naturally occurring iso-agglutinins existed that the serum of any member of this group must not be able to agglutinate another member of the same group. With this limitation in mind, it was found to be necessary to split this fourth category into two separate groups (Table XLVI).

The platelets having been subdivided into five separate groups, it was possible to suggest a theoretical phenotype and genotype for each platelet groups and determine a percentage incidence for each group. The letters X Y Z and o were used to designate these groups (Table XLVI).

The technique used in arriving at these conclusions was essentially similar to that used by Landsteiner (1900) when he discovered the existence of the ABO blood group system in human blood.

TABLE XLV

An analysis of positive platelet agglutination tests unexplained in terms of ABO incompatibility. (Data derived from Fig. 22 and Table XXXVIII).

Identification No. of Donor of Platelets or Serum	Platelets Agglutinated by serum derived from donors numbered:-	Serum Agglutinated Platelets from donors numbers:-
1	-	-
2	5, 22	29
3	-	-
4	-	-
5	10	2
6	11, 39	-
7	-	-
8	-	-
9	-	12
10	-	5
11	-	6, 15
12	9, 14	17
13	30	15
14	-	12, 20
15	11, 13, 17	-
16	-	20
17	12	15
18	37	19, 30
19	18	-
20	14, 16	31
21	26	-
22	-	2
23	27	-
24	26	-
25	-	-
26	-	21, 24
27	-	23, 34
28	-	34, 38
29	2	-
30	18	13
31	20	-
32	37	35
33	-	-
34	27, 28	-
35	32	38, 40
36	-	-
37	-	18, 32
38	28, 35	-
39	-	6
40	35	-
TOTAL	29	29

Table XLVI

Theoretical Platelet Group Structure derived from Fig.22 and Table XLV

Category (p.	Identification Numbers of Platelet Donors (Table)	Theoretical Platelet Group	Theoretical Genotype	Theoretical Iso Agglutinin	Percentage Distribution
1	1, 3, 4, 7, 8, 25, 33, 36	o	oo	Nil	20%
2	9, 10, 11, 14, 16, 20, 22, 26, 27, 28, 37, 39	X	XX, Xo	Anti Y Anti Z	27.5%
3	6, 15, 19, 21, 23, 24, 29, 31, 34, 38, 40	YZ	YZ	Nil	27.5%
4	2, 12, 13, 18, 20, 32	Y	YY, YX, Yo	Anti Z	15%
	5, 17, 30, 35	Z	Zz, ZX, Zo	Anti Y	10%

The letter small o was applied to Group 1 where neither antigen or antibody appeared to be present, the letter X to Group 2, the letters YZ to Group 3 and the letters Y and Z to the two sub-divisions of Group 4 (See p. and Table)

The evidence provided by these experiments allowed this theoretical platelet group structure to be evolved but, by itself, this was insufficient evidence to base a definite group structure. As Race & Sanger (1954) have suggested, further elucidation of any blood group system may be obtained by preparing appropriate antisera in animals. Therefore, two rabbits were immmnised by serial intravenous injections of platelet suspensions derived from donors whose theoretical platelet groups were o and Z. Antisera from these animals were then tested against platelet suspensions from 20 normal donors selected at random to see if any selective cross agglutination could be demonstrated (Experiment 42. Appendix B, p. 32). Both sera, however, agglutinated all 20 platelet suspensions while sera from normal non-immunised rabbits had no such agglutinating powers (Table XLVII).

This did not confirm the subdivisions made in Table XLVI as, if the results had been as expected, the rabbit receiving the Group o platelet suspensions, should not have produced any antibody while the other receiving the Group Z platelet suspension, should have only been able to agglutinate 9 of the suspensions tested (namely those suspensions included in Group Z and YZ).

This cast considerable doubt on the existence of separate platelet groups, but it is possible that these anti-human platelet rabbit sera may have contained a non-specific agglutinating factor which masked any specific group antibodies.

Table XLVII Experiment 42 (Appendix B, p32)

Platelet agglutination tests with specific anti-human platelet rabbit serum.

Rabbit Serum	Theoretical Antibody	Platelet agglutination																			
		Platelet Suspension Number (See tables XXXVIII & XLV & XLVI)																			
		6	4	15	11	14	28	35	38	40	39	18	32	34	30	22	27	29	38	37	2
1	Anti o or no antibody	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Anti Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3 Control	Nil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Titre of rabbit serum against human platelets.

1. 1/256
2. 1/64
3. Nil

By selective adsorption of similar sera with platelets it may be possible to determine whether this is so.

This theoretical exercise has provided a basis for carrying out further cross absorption experiments between sera and platelet suspensions in an attempt to confirm or refute the presence of a separate platelet group structure and group specific antibodies. This, together with further cross agglutination experiments using serum derived from cases of idiopathic thrombocytopenia, may eventually solve this problem.

Although similar experiments have been done by both Stefanini et al (1953b) and Harrington et al (1953), their results can be criticised on the grounds that the sera they used, in certain of their experiments, contained platelet agglutinins stimulated by repeated blood transfusions given to combat the anaemia and thrombocytopenia in patients with aplastic bone marrows. The antigenic structure of the platelets transfused in these cases was naturally unknown and therefore the sera obtained from such patients could not possibly contain antibodies that were group specific. When Stefanini and his co-workers applied their theoretical concept of platelet groups to transfusion studies in cases of thrombocytopenic purpura, they found that compatible platelets did not survive any longer than incompatible suspensions (Stefanini et al 1953b).

Yet the observations that repeated platelet transfusions of ABO and Rhesus compatible blood appear to stimulate anti-

platelet factors in the blood of thrombocytopenic patients (p.115) and that neonatal thrombocytopenia can occur in children of healthy non-thrombocytopenic mothers who have specific platelet iso-agglutinins in their blood (p. 124), do provide strong evidence to support this hypothesis of separate platelet groups. While this clinical evidence suggests that separate platelet groups do exist, it has been impossible, so far, to provide irrefutable serological proof of their existence.

Whatever the implications of this work, one practical fact emerged. If sera from a wide series of cases were to be examined for platelet agglutinating factors, the platelet suspensions should be derived either from ABO compatible blood, or from Group O blood alone. Therefore in the tests to be described in the following chapter, platelet suspensions were prepared from Group O red cell donors, irrespective of the group of the blood from which the test serum was derived.

CHAPTER XII

Platelet Agglutination in Thrombocytopenic and Non-Thrombocytopenic Purpura

Serum was obtained from cases with both thrombocytopenic and non-thrombocytopenic purpura and from any case where in vivo platelet agglutination might provide an explanation for the signs and symptoms. The test was performed in every case as detailed in Appendix A, p. 14. Each serum was tested against at least four platelet suspensions derived from Group O blood and if positive, the titre of the agglutinating factor was determined against one of the platelet suspensions agglutinated in the initial test.

A positive result was recorded if the test serum agglutinated at least two separate suspensions of platelets (Appendix A, p. 16). When using platelets derived from Group O blood, 2.55% of normal sera gave positive results, and therefore this criterion of a positive result reduced the probability of a false positive result. The sera from 73 cases were tested against normal platelet suspensions and 29 of them gave positive results. An analysis of these positive results was made in order to determine in what types of cases positive tests occurred (Table XLVIII).

The relevant details of all 73 cases have been summarised in Table LV and summaries of the case histories given in Appendix C.

Table XLVIII

Distribution of Positive Agglutination Tests

In Cases with Thrombocytopenia		
Diagnosis	Number Tested	Number of Positive Agglutination Tests
Acute Idiopathic Thrombocytopenic Purpura	9	1
Chronic Idiopathic Thrombocytopenic Purpura	18	11
Idiopathic Thrombocytopenic Purpura (unspecified duration)	4	1
Secondary Thrombocytopenia	8	1
Drug Thrombocytopenic Purpura	4	2
Neonatal Thrombocytopenic Purpura	5	4
Cryoglobulinaemic Purpura	2	2
Acquired Haemolytic Anaemia with associated Thrombocytopenia	2	1
Acute Disseminated Lupus Erythematosus	1	0
Defibrination Syndrome of Pregnancy	2	0
TOTALS	55	23

Percentage of positive results = 41.85%

In Cases with Normal Platelet Counts		
Cryoglobulinaemic Purpura	1	1
Henoch Schonlein Purpura	4	0
Virus Pneumonia	1	1
Acute Disseminated Lupus Erythematosus	1	0
Unspecified Group of Non Thrombocytopenic Purpura	11	4
TOTALS	18	6

Percentage of positive results = 33.3%

The results have been analysed under the following headings:-

(1) The incidence of positive agglutination tests in cases of thrombocytopenia and in those with normal platelet counts.

(2) The incidence of positive tests in the several types of thrombocytopenia.

(3) The incidence of splenomegaly in those cases with positive tests as compared with those with negative tests.

(4) The relationship of blood transfusion or pregnancy to the positive agglutination tests.

(5) The relationship of positive agglutination tests to therapy (steroid administration and splenectomy).

These positive tests were a measure of the power of serum derived from these cases to agglutinate normal platelet suspensions and, if the agents responsible were acquired immune agglutinins, they must have been, by definition, iso-agglutinins. The ability of the sera to agglutinate platelets derived from the same blood (auto-agglutinins) was only tested in those cases with normal platelet counts, as it was found to be impossible to prepare satisfactory suspensions of platelets from the blood derived from cases of thrombocytopenia, even when using large quantities of blood. The platelets from thrombocytopenic individuals did not resuspend satisfactorily when separated from their plasma, a

fact which in retrospect may be significant (see p. 185). This difficulty was also encountered by Nolthemius et al (1953). However, if a case with thrombocytopenia is found to have a platelet agglutinating factor in the blood that will agglutinate platelet suspensions derived from normals, it is not unreasonable to suppose that it must also be capable of producing agglutination of the platelets, and consequent thrombocytopenia in the blood from which it was derived.

I. The Incidence of Positive Agglutination Tests in Thrombocytopenic and Non-Thrombocytopenic Purpura.

An analysis of all the tests performed showed that the proportion of positive results was the same whatever the platelet count.

TABLE XLIX

Type of Case	Number Tested	Positive Agglutination Tests	Negative Agglutination Tests
Thrombocytopenic	55	23	32
Non-thrombocytopenic	18	6	12

An analysis of these results (Appendix D, p. 4) shows that P is greater than 0.7 and therefore there was no significant variation between these two groups.

II. The Incidence of Positive Tests in Different Types of Thrombocytopenia

Acute and Chronic Thrombocytopenic Purpura

Table XLVIII illustrates clearly that a higher percentage of positive results occurred in chronic idiopathic thrombocytopenia than in the group of acute idiopathic

thrombocytopenia. (Cases with symptoms of more than 6 months were classified as chronic). These results, however, agree with those of Sauer & Van Loghem (1954) and Stefanini (1955b) who also determined a higher incidence of positive tests in cases of long duration.

Among those cases classified as chronic thrombocytopenic purpura are four cases which, clinically, were considered to be examples of hypersplenism. All had anaemia, leucopenia, and thrombocytopenia secondary to splenomegally of unspecified aetiology. As the cause of the splenomegaly and the pancytopenia was unknown, and the duration of the illness was prolonged, they were thought to justify this grouping.

Secondary Thrombocytopenia

In secondary thrombocytopenia due to replacement of bone marrow with secondary malignant tumour deposits or due to uncontrolled proliferation of the constituent cells, only one case was found to have positive agglutination test (Case 46).

Drug Induced Thrombocytopenic Purpura

Several cases were encountered in which a drug or other toxic agent may have been responsible for reducing the platelet count and inducing purpura.

In two, the offending drug was ascertained by in vitro platelet agglutination tests and the sensitivity confirmed after the platelet count had returned to normal on withdrawal of the drug therapy. The techniques used are described in Appendix A, p.16. Case 36 showed definite sensitivity to

sulphamezathine and, what was more interesting, showed a definite cross sensitivity reaction with other members of the sulphonamide group. The purpura apparently resulted from the administration of sulphamezathine but, on in vitro testing, positive results were obtained with sulphathiazole and sulphanilamide as well as sulphamezathine. This was surprising in view of the definite specificity of the chemically related drugs Quinine and Quinidine in 'in vitro' tests when one or other of these drugs has been implicated as the causal agent in thrombocytopenic purpura (Bolton & Young 1953; Bolton 1956).

Case 35 showed a definite sensitivity to the antihistamine drug 'Histantin' (Chlorocyclazine Hydrochloride) which was being administered along with Carbrital and Codeine. This provided yet another example of an antihistamine preparation which is capable of inducing thrombocytopenia, previous examples having been cited by Ackroyd (1955b) (Antazoline), and Harrington, Minnich & Arimura (1956) (Benadryl). Two further cases were placed in this group although in both instances they were not confirmed by in vitro testing.

In Case 38, a definite episode of thrombocytopenia was induced by skin testing with Johnston's Furniture Polish. The nature of this preparation rendered it impossible to perform any in vitro tests.

In Case 37, thrombocytopenia, associated with severe agranulocytosis, was induced by uncontrolled administration of

methyl thiouracil. Blood from this case gave entirely negative in vitro tests for platelet sensitivity to this drug. On withdrawal of the drug, the white cell and platelet count returned to normal levels. The bone marrow biopsy in this case showed marked toxic changes with maturation arrest of myelopoesis, megaloid erythropoesis and apparent absence of megakaryocytes. Thus this may not have been an example of an immune reaction but rather of toxic depression of marrow activity by the drug. Drugs of the thiouracil group have rarely been implicated as instigators of thrombocytopenia (Moore 1946).

In three other cases symptoms associated with thrombocytopenia have followed the recent administration of drugs (Quinine, Aspirin, Phenacetin, Camphor, Codeine, Benzocaine, Cocaine, Penicillin) but in none of these did in vitro tests confirm any definite association between the drug in question and platelet sensitivity. As none of these cases recovered spontaneously following withdrawal of these drugs, they were grouped as cases of idiopathic thrombocytopenia according to the duration of the thrombocytopenia.

Further cases of non-thrombocytopenic purpura associated with drug therapy will be considered under the non-thrombocytopenic group.

Neonatal Thrombocytopenia. (Cases 30, 31, 32, 33, 34).

Five cases of neonatal thrombocytopenia were encountered in infants in the first few days of life. All these

children showed either purpura or some other evidence of spontaneous haemorrhage associated with thrombocytopenia. In none could any evidence of ABO or Rhesus incompatibility be proved. Three combinations of thrombocytopenia in pregnancy have been described (see p.125).

(1) The occurrence of thrombocytopenia in both mother and child;

(2) Thrombocytopenia in the mother but a normal child;

(3) Normal mother with a thrombocytopenic child.

All five cases tested fell into the third group. Four of the mothers had never at any time shown any abnormal bleeding tendency nor were they ever found to be thrombocytopenic. One mother (Case 31) developed a transient purpuric rash at 38 weeks of pregnancy. This was confined to her waist and disappeared in 2 days but at no time was she found to be thrombocytopenic. In four, a platelet agglutinating factor could be demonstrated in the mother's serum. In three cases this factor did not agglutinate the mother's own platelets but only those of other normals including, in the two instances tested, their husband's. These cases were therefore typical examples of this group.

The fourth case gave unusual results, in that although the mother appeared to be entirely normal, her serum agglutinated her own platelets, as well as her husband's and those of normals. This was observed twice following the delivery of two successively affected children (Case 34).

In the fifth case (Case 33), no agglutinating factor could be demonstrated in the mother's serum although the child was definitely thrombocytopenic. The thrombocytopenia in this child, however, was very short lived (1 week) unlike that seen in the other 4 cases, where it persisted for between 6 - 8 weeks. In three cases, where a platelet agglutinating factor was present in the mother's serum, the first-born child had been affected, although none of the mothers had had previous miscarriages or blood transfusions. If this syndrome is, as suggested, the result of the mother acquiring a specific immune platelet agglutinin, the exact method of sensitisation is difficult to explain. In the analogous state, due to red blood cell Rhesus factor incompatibility between mother and child, the mother requires the stimulus of at least one previous pregnancy, miscarriage, or blood transfusion, before she will produce specific immune antibodies. Therefore first-born children are virtually never affected unless the mother has had a previous miscarriage or received an incompatible blood transfusion. In cases where ABO incompatibility between mother and child has resulted in haemolytic disease of the newborn, first-born infants are frequently affected. In such cases the naturally occurring antibody in the mother's blood is stimulated rapidly by the foreign foetal blood group antigens and, following trans-placental transfer, may produce haemolysis of the red cells in the first-born child (Mollinson 1951).

If the analogy of platelet group incompatibility is to be sustained as an explanation for cases of neonatal thrombocytopenia arising in first-born children of normal mothers, it must be assumed that the mothers of these cases had had naturally occurring platelet antibodies in their blood, which were raised to abnormally high levels by the stimulus of the foetal foreign platelet antigen. On transplacental transfer, this antibody had, in turn, damaged or destroyed the foetal platelets and in some instances, provoked purpura. Therefore it is not unreasonable to suppose that the mothers of these thrombocytopenic first-born children, by virtue of having naturally occurring platelet antibodies, destroyed the platelets in their children as these antibodies rose to very high titres.

The duration and behaviour of the thrombocytopenia in the infants closely simulates that seen in Erythroblastosis Foetalis and the common duration of the thrombocytopenia (5 - 6 weeks) agrees closely with the half life of immune globulin, namely 20 - 30 days (Tullis 1956). The presence of an 'in vitro' platelet agglutinating factor in the serum of one mother (Case 34) cannot be explained by any current theory. Although this was capable of agglutinating the mother's own platelets in vitro, it had not produced any thrombocytopenia or otherwise damaged her platelets.

Thus, while certain discrepancies were found in this series of cases which did not confirm the accepted theories of

the pathogenesis of neonatal thrombocytopenia, they provided insufficient evidence to justify any radical change of opinion. The demonstration of a platelet agglutination factor in the blood of a pregnant mother may be of practical importance and the implications of a positive finding in these cases will be further discussed on p.200.

Defibrination Syndrome of Pregnancy

Two cases in whom a severe lack of fibrinogen, complicating the terminal stages of pregnancy, were discovered to be also severely thrombocytopenic. In Case 49, the prolonged carrying of a dead foetus in utero provoked a severe haemorrhagic state in the mother, which, when found to be associated with marked thrombocytopenia, was originally thought to be acute idiopathic thrombocytopenic purpura. On subsequent examination the lack of fibrinogen was discovered and adequate therapy with fibrinogen produced a dramatic improvement in the patient's condition and a cessation of all haemorrhage. The platelet count, however, did not return to normal for 10 days.

In the second (Case 48), a concealed accidental haemorrhage provoked defibrination and severe thrombocytopenia. Again fibrinogen therapy improved the haemorrhagic state without producing a rise in the platelet count. The latter returned to normal 5 days later.

In neither case could any platelet agglutinating factor be demonstrated in the serum obtained before or after the

administration of fibrinogen.

The pathogenesis of this syndrome is generally accepted to be due to massive release of tissue thromboplastin from the placenta or decidua into the systemic circulation with resultant intravascular fibrin formation and depletion of the total blood fibrinogen (Schneider 1955). Presumably the platelets become involved in this mechanism and are depleted during the in vivo coagulation of the blood by undergoing VM by becoming entrapped by the fibrin as it forms. Therefore, at present, there is no evidence that any specific platelet agglutinating factor, other than those which may produce VM during intravascular coagulation, exists in these syndromes.

Acquired Idiopathic Haemolytic Anaemia and Thrombocytopenia

In two instances thrombocytopenia and haemorrhagic signs had arisen in cases of acquired haemolytic anaemia of long standing. In both cases the red blood cells were found to be coated with incomplete antibodies by the direct Coombs test.

Only in one cass (Case 51) could any platelet agglutinating factor be demonstrated and that disappeared from the blood following 1 week's Prednisone therapy.

The positive agglutination test obtained in this case may well have been the result of multiple blood transfusions. This patient, besides having a non-specific red cell agglutinin of the cold antibody type, had an immune iso-agglutinin against S+ red cells, her own red cells being S-. Platelets

from donors, whose red cell group was S-, were agglutinated by serum from this case as well as platelets from S+ donors, thus the platelet agglutinating factor was not related to this specific antibody.

The second case (Case 50) in spite of having a positive Direct Coombs Test on his red cells did not have any demonstrable platelet agglutinating factor. The serum tested was prepared before the start of successful steroid therapy. No specific red-cell group antibody was detected in this case.

This combination of haemolytic anaemia and thrombocytopenia originally led Evans & Duane (1949) to revive the concept of an immune mechanism in idiopathic thrombocytopenia. That such a mechanism existed was confirmed in one of these cases, where an active platelet agglutinating factor was demonstrable against several platelet suspensions. No valid explanation can be offered for the failure to demonstrate a platelet agglutinating factor in Case 50.

Non-Thrombocytopenic Purpura

Eighteen cases of purpura or unexplained abnormal bleeding tendency were encountered in which the presence of in vivo platelet agglutination might have provided a rational explanation for the observed clinical state. The platelet count was never found to be other than within normal limits in these cases. It is impossible to exclude that transient thrombocytopenic did not exist for a very short time in these cases, and that during this phase, purpura or other haemorrhagic

manifestations may have occurred. Ackroyd (1952) found that when he administered a test dose of a drug to a patient with established sensitivity to that particular drug, the peripheral platelet count fell to very low levels within 2 hours. Similarly, the administration of bovine antihaemophilic globulin to patients with haemophilia will induce thrombocytopenia within a similar time and the platelet count returns to normal levels within a few hours (Macfarlane, Biggs & Bidwell 1954). Earlier studies by Achard & Aynaud (1908 a, b & c) showed that similar transient episodes of thrombocytopenia could be induced in experimental animals by the intravenous injection of gelatine or peptone. So it is possible that many cases of so-called non-thrombocytopenic purpuras would have been found to be thrombocytopenic if examined at the appropriate time.

Henoch Schonlein Purpura

Four cases of non-thrombocytopenic purpura were encountered which were clinically considered to be examples of the Henoch Schonlein group. No positive platelet agglutination tests were found in any of these cases.

Cryoglobulinaemic Purpura

Three cases of severe purpura associated with cryoglobulinaemia were encountered, of which two were thrombocytopenic. In all three, the abnormal protein fraction or cryoglobulin could be demonstrated in abnormal amounts by its property of spontaneous flocculation at room temperature

or at 4°C. All three cases appeared to fall into the group of essential cryoglobulinaemia described by Lerner & Watson (1947), and Waldenstrom (1952). The cryoglobulins were in each case discovered in the plasma or serum of these patients and were not associated with other abnormal proteins. (Macroglobulins or a high gamma-globulin fraction). In no instance was there any evidence of leukaemia or multiple myelomatosis.

Platelet agglutination tests were positive in all these cases at those temerpatures which allowed the cryoglobulins to spontaneously precipitate namely room temperature and 4°C. If the serum and added platelets were kept at 37°C no agglutination occurred.

This, therefore, would appear to be an expression of non-specific platelet agglutination associated with the spontaneous precipitation of the cryoglobulin fraction of the serum proteins, rather than a measure of any specific platelet agglutinating factor. It is of interest to know that such platelet agglutination may occur in these cases as the cryo-protein precipitates. The severe purpura, skin ulceration and Reynaud's phenomenon, that these cases may develop on exposure to cold (Waldenstrom 1952), may be due to a similar mechanism taking place with the blood vessels in vivo.

Acute Disseminated Lupus Erythematosus

Two cases of this syndrome were encountered where the

clinical diagnosis was confirmed by in vitro demonstration of the Lupus Erythematosus cell phenomenon. In neither case could any platelet agglutinating factor be demonstrated.

Positive agglutination tests have, however, been reported in this syndrome by Harrington et al (1956).

Unspecified Non-Thrombocytopenic Purpura

In the assorted series of cases which were grouped as unspecified non-thrombocytopenic purpura, four cases were found to have platelet agglutinating substances in their sera. In all four the sera contained factors capable of agglutinating both the patient's own platelets and those of normals.

Case 64 had received multiple transfusions of compatible blood to replace blood lost per rectum in an acute exacerbation of ulcerative colitis. Following one transfusion of 2 pints of blood she developed localised purpura on the anterior chest wall and shoulder. On testing, a platelet agglutinating factor was found to be present in her blood which agglutinated both her own platelets and those of normal donors. Her platelet count was always normal and, when ~~tested~~ examined 3 weeks later, the platelet agglutinating factor could not be demonstrated.

Case 58 had received over 100 blood transfusions for unexplained gastrointestinal haemorrhage and although he was never found to be thrombocytopenic or to have purpura, his blood contained a platelet agglutinating factor against his own platelets and those of certain normals.

These cases were probably examples of a platelet agglutinin developing as a result of repeated blood transfusions. The relationship of blood transfusion to platelet agglutinating factors will be discussed further on page 170.

Case 65 developed purpura following a reaction to the administfation of several pints of human albumin intravenously. He too was found to have a weak platelet agglutinating factor in his serum which agglutinated both his own platelets with those of normals. This reaction could be enhanced by the addition of reconstituted dried human albumin (Lister Institute) but not by a preparation of his own albumin prepared two weeks after his last transfusion of albumin (Experiment 44. Appendix B p.35.) As in previous cases, no drop in the platelet count was ever demonstrated.

The latter case appeared to be an example of a reaction to human albumin which had acquired antigenic properties in this particular case and had stimulated a platelet agglutinating factor. The observation that the patient's own albumin, freshly prepared, did not provoke a similar reaction in vitro suggested that either the human albumin had been denatured during the process of preservation or that it contained some other unspecified antigenic material common to both the transfused albumin and the albumin used to prepare solutions for in vitro testing. The mechanism of antibody production, in this case, may have been similar to that suggested by Ackroyd in drug purpura. Alternatively, an unrelated antigen-antibody reaction may have produced transient

thrombocytopenia and purpura as suggested by Hoigne, Grossman & Stork (1955) and Miescher & Straessle (1956).

In one isolated example (Case 72) a powerful platelet agglutinating factor was discovered in the serum of a case convalescent from acute virus pneumonia. This had entirely disappeared three weeks later. Again no purpura or thrombocytopenia was ever discovered.

On four occasions cases have been submitted for investigation following the appearance of purpura during a course of multiple drug therapy or following the isolated administration of one specific drug. None of these cases were thrombocytopenic nor could sensitivity to these drugs be demonstrated in vitro by the techniques described in Appendix A, p.16. The pathogenesis of these purpuric episodes was never satisfactorily determined.

CHAPTER XIII

The Significance of the Positive Agglutination Test in Thrombocytopenia

The Relationship of Positive Agglutination Tests to Splenomegaly

It has been often suggested that any platelet agglutinating factor might induce thrombocytopenia by accelerating the destruction of platelets by the spleen. Conversely the role of the spleen might be to produce the agglutinating factor which either damages the platelets of the peripheral blood or inhibits their formation from the megakaryocytes in the marrow (Troland & Lee 1938; Dameshek & Miller 1946).

Whatever the explanation, the results obtained in this series of tests were analysed to see if there was any specific correlation between positive agglutination tests and splenomegaly (Table L).

TABLE L

Platelet Agglutination Tests and Splenomegaly.

	Positive Tests	Negative Tests
Normal spleens	15	36
Enlarged spleens	9	11

Statistical analysis (Appendix D, p. 4) showed that P was greater than 0.30 and therefore the difference between these groups was not significant and was most likely due to chance.

Platelet Agglutination Tests and Response to Therapy in Thrombocytopenic Purpura

It has always been hoped that the platelet agglutination tests would be of value in assessing prognosis or response to therapy in cases of thrombocytopenia, and, in several series of cases, analyses of results have been made to decide whether cases with positive tests had responded more favourably to therapy than those with negative tests. Harrington (1954) claimed that this was true in relation to splenectomy. In his series of cases he found that those cases of thrombocytopenia with positive tests responded more favourable to splenectomy. His opinion was supported by Bernard & Mathe (1955). But Stefanini (1955) in an analysis of his series denied this association, as did Tullis (1956).

Although the present series of cases is smaller than those of other workers, an analysis of the response to therapy and positive platelet agglutination tests was thought to be worth while. 22 cases with thrombocytopenia received steroid therapy. 12 showed a complete or partial response and 10 showed no response. (Table LI).

TABLE LI

Steroid Therapy

Platelet Agglutination Test	Total Number	Response	Negative Response
Positive	7	4	3
Negative	15	8	7

Inspection of these results showed that the differences were unlikely to achieve significance (Appendix D, p. 5). Therefore these results did suggest that a positive platelet agglutination test provided no guide to the response to steroid therapy in thrombocytopenic syndromes.

TABLE LII

Splenectomy

12 cases had their spleens removed. 7 responded and 5 showed a complete or partial return to normal.

Platelet Agglutination Test	Response	No Response
Positive	4	2
Negative	3	3

One further case died immediately after splenectomy before the effect of the operation could be assessed.

Again, inspection of the results in Table LII show that the differences between these groups were unlikely to achieve significance (Appendix D, p. 5), and therefore, a positive agglutination test was no guide to the outcome of splenectomy in cases of thrombocytopenic purpura.

The Relationship of Blood Transfusions and Pregnancy

It has been stated by Harrington (1955) that previous pregnancy or blood transfusion may themselves produce a platelet agglutinating factor in the blood and thus induce thrombocytopenia. Certainly in cases of secondary thrombo-

cytopenia, blood or platelet transfusions have been shown to do this, and subsequent platelet transfusions have become, as a result, progressively less beneficial.

It has been shown (Table XLVIII) in this present series, that more positive agglutination tests occurred in the chronic or long standing cases of thrombocytopenia and it was possible that blood transfusion given to combat the haemorrhagic tendency in long standing thrombocytopenic purpura, or to correct anaemia, might have provoked a platelet agglutinating factor, thus preventing a spontaneous remission.

The present series of cases have been analysed to determine whether either previous blood transfusion or pregnancy were more common in those cases with positive agglutination tests than those with negative tests (Table LIII A & B). Statistical analyses of these results showed that the differences between the groups in these two surveys were not significant (Appendix D, p.6). Therefore neither pregnancy nor blood transfusion appear to be capable of producing a platelet agglutinating factor. This does not exclude that they may be the stimulus in certain individual cases and, as has been already discussed, multiple transfusions may have been responsible for the appearance of a platelet agglutinating factor in 3 cases (51, 58, 64) in the present series.

TABLE LIII A

Blood Transfusion and Platelet Agglutination

	Platelet Agglutination Test	
	Positive Tests	Negative Tests
Previous Blood Transfusion	10	13
Never Received Blood Transfusion	19	29

TABLE LIII B

Pregnancy and Platelet Agglutination Tests

	Platelet Agglutination Test	
	Positive Tests	Negative Tests
Previous Pregnancy	15	14
Never Pregnant	14	30

Physical Properties of the Platelet Agglutinating Factor

The properties of the platelet agglutinating factor found in certain cases of this series were compared with those of the factor found in fresh sera which could induce agglutination and VM in washed platelets. A high titre serum (Case 8) was selected for testing. Initially it was treated by heat and absorption with barium sulphate as all the sera in this series. The continued ability of this serum to induce platelet agglutination after various treatments, additions, and fractionations was observed (Table LIV).

These observations showed that the platelet agglutinating factor had certain well defined properties which separated it from the known blood coagulation factors remaining in fresh serum after clotting (Compare Table XIII).

(1) This factor resists heating at 56°C for 1 hour but is destroyed by heating at 65°C for 15 minutes.

(2) It is relatively stable on storage and may be preserved for long periods at -15°C.

(3) The agglutinating power was partially inhibited by the decalcifying salts sodium oxalate and disodium versene, but not by trisodium citrate.

(4) The factor is adsorbed from the serum by concentrated platelets but not by red cells or white blood cells. Insufficient serum was available to see if any selective

adsorption took place when using a large number of separate platelet suspensions. This, if it had been possible, might have given further information as to the occurrence of platelet groups.

(5) The 33-50% ammonium sulphate fraction of the serum, but no other fraction, retained the original agglutinating factor.

(6) The Euglobulin fraction of serum did not show any activity.

TABLE LIV Experiment 42 Appendix B, p. 32

The physical properties of platelet agglutinating factor.

(1) 0.2 ml serum containing proven platelet agglutinating factor was added to 0.2 ml washed platelet suspension together with 0.1 ml addition. (2) 0.2 ml serum was treated by various adsorbents or anticoagulants before adding to 0.2 ml washed platelet suspension. Both systems were then incubated for 2 hours at 37°C and then the presence or absence of platelet agglutination recorded. In each instance the serum was used undiluted and treated by heat 56°C for 30' and barium sulphate absorption prior to testing.

Platelets	Serum $BaSO_4$ Absorbed	Addition and Concentration	Presence or Absence of Platelet Agglutination
Normal washed	Test serum	Saline 0.85%	+
"	Heated 65° 15'	"	-
"	Stored 24 hrs. R.T.	"	+
"	Stored 7 days R.T.	"	-
"	Stored 21 days 4°C	"	+
"	Stored 3 mos. -15°C	"	+
"	Dialysed test serum	"	+
"	Test serum	Na Citrate 3.8% (1 part to 9 parts serum)	+
"	"	Na Oxalate 1.34% (1 part to 9 parts serum)	±
"	"	Na_2 Versene 4.5% (1 part to 33 parts serum)	±
"	Test serum	Heparin 20 u/ml	+
"	"	Thrombodym 2.5%	+
"	Platelet absorbed test serum	-	-
"	Red cell absorbed serum	-	-
"	25% Ammonium Sulphate fraction of test serum	-	-
"	25-33% Ammonium Sulphate fraction of test serum	-	-
"	33-50% Ammonium Sulphate fraction of test serum	-	+
"	Euglobulin fraction of test serum	-	-

CHAPTER XIV

Alternative Techniques for Demonstrating Anti-Platelet Factors in Blood

Complement Fixation Test (Appendix A, p.21).

A large number of techniques have been described which were thought to measure either a platelet agglutinating factor, or other anti-platelet factors, in the blood obtained from certain patients with idiopathic thrombocytopenic purpura. It has been assumed by nearly every worker in this field of immuno-haematology that these factors are immune antibodies, the majority being specific platelet agglutinins.

Tullis (1953 & 1956) and Bessis & Tabuis (1955) have claimed that certain of these antibodies were lysins and required Complement for their action. The platelet-agglutination technique used in the present series of tests would not be expected to demonstrate such platelet lysins, as the sera were inactivated, and their Complement destroyed before testing. If platelet lysins requiring Complement were present in these sera, it was possible that their presence could be demonstrated by their power to utilise or fix Complement as they reacted with platelets. Therefore a Complement fixation test was designed in an attempt to demonstrate these specific platelet lysins in the hope that

such a test might provide a more sensitive method for detecting anti-platelet factors in blood or serum (Appendix A, p.21).

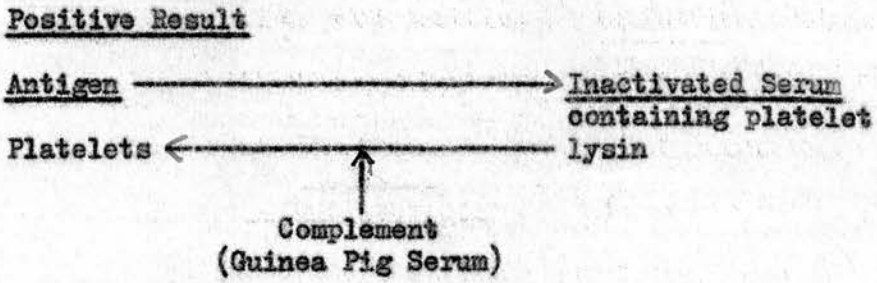
It has been shown in Chapter IV that when fresh serum induces VM in platelets, Complement is not fixed or consumed. Therefore any change of Complement levels in serum when mixed with platelets would be a significant finding and might be a measure of a specific platelet lysin.

Washed normal platelet suspensions were used as the antigen and Complement was provided in the form of guinea pig serum. The human serum to be tested was inactivated to destroy its natural Complement prior to incubation with platelets. The antigen, Complement, and test serum were incubated together for a fixed period. Then the platelets have been removed, the titre of residual Complement was determined. This titre was compared with that of control mixtures.

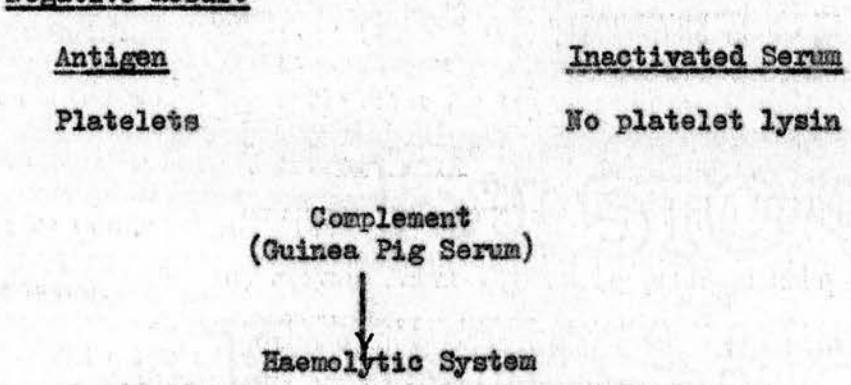
This technique was preferred to more orthodox methods as the platelet suspensions used as antigen showed varying anti-complementary activity, presumably due to trace amounts of adsorbed disodium versene, used as the anticoagulant to facilitate the separation of the platelets from the blood. This anticoagulant removes magnesium as well as calcium from the blood and thus inhibits the ability of Complement to react with the haemolytic system.(Mayer, Croft & Gray 1948). The test may be diagramatically represented in Fig. 24.

FIGURE 24

Complement Fixation Test

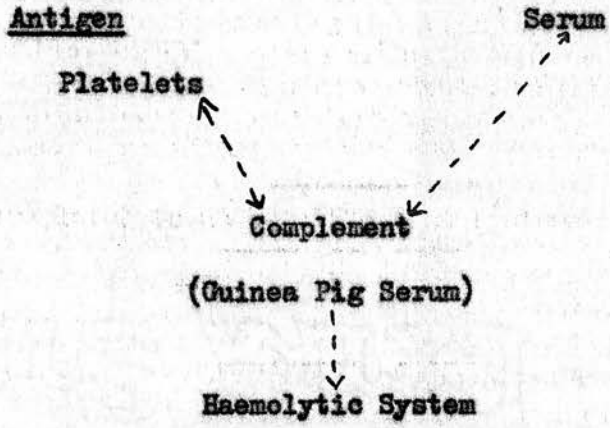


Complement fixed by reaction of platelet lysin with platelets and therefore no haemolysis of sheep cells can occur.



Complement not fixed by serum antigen reaction and is available for the haemolytic system. Haemolysis therefore occurs.

Anticomplementary Reaction



Either the serum or platelets used in the test system may destroy Complement partially or completely, and therefore haemolysis is inhibited correspondingly.

20 of the sera tested in the previous series, including 5 sera which gave positive agglutination tests, were tested, along with 20 normal sera, for their ability to fix Complement when incubated with platelets. In addition, sera from two rabbits containing potent anti-human platelet agglutinins and lysins were tested along with two samples of normal rabbit serum (Appendix A, p.23).

In this test none of the twenty human sera showed any evidence of fixing Complement in the presence of normal human platelets, whereas both the anti-human platelet rabbit sera were able to fix Complement as they reacted with human platelets.

These findings confirm those of De Nicola, Rosti & Zangaglia (1955) and Stefanini (1955b) and Introzzia & De Nicola (1956).

Mixed Red Cell Platelet Agglutination Test for the Detection of Antibodies to Human Platelets

(Coombs, Marks & Bedford 1956). (Appendix A, p.24).

Following the successful use of this technique to confirm the ABO antigens on platelets it was decided to determine whether the sera, encountered in this series and which gave positive direct agglutination test, would also give positive results with the above technique. 2 sera with high titres 1/8 and 1/32 respectively were tested together with 2 normal sera. All the sera were heat inactivated and barium sulphate absorbed as for the direct agglutination test.

The sera were first incubated as for the platelet agglutination test with a suspension of washed normal human platelets and incubated for 1 hour at 37°C. The platelets were then washed, resuspended and an equal volume of Group O Rhesus positive red cells, sensitised with incomplete Anti-D

and one volume of anti-human globulin serum were added to each suspension. This mixture was incubated at 37°C for 30 minutes and then spun at 2400 g. for 2 minutes and the cells resuspended.

The preparations were then examined for the presence of mixed red cell platelet agglutination.

Results

In no instance did mixed red cell/platelet agglutination occur but with both test sera and normal sera, separate clumps of agglutinated platelets and agglutinated red cells were visible.

These results were similar to those obtained by Coombs and his co-workers (1956).

Tanned Red Cell-Platelet Protein Agglutination Test

(Kissmeyer Nielsen 1953) (Appendix A, p. 18)

Kissmeyer Nielsen (1953) adapted the use of the tanned red cell-protein coupling evolved by Boyden (1951) to demonstrate that the presence of anti-platelet factors in the blood of patients with idiopathic thrombocytopenic purpura. In this technique platelet protein obtained by lysing pure platelet suspensions was allowed to combine with red cells tanned with tannic acid. Test sera were added to these coated cells and Kissmeyer Nielsen claimed that if any anti-platelet factor was present in these sera the tanned and coated red cells would be agglutinated. By means of this method he claimed that he was able to demonstrate anti-platelet agents in the blood of certain cases of thrombocytopenic

purpura. His claims were supported by the results of Verstraete and Vandenbroucke (1955), and Sauer & Van Loghem (1954).

11 of the sera in this present series, together with 10 normal sera, were tested using this technique.

While the occasional positive results were obtained, this was also true of the normal controls. Furthermore, while tanned red cells by themselves did not spontaneously agglutinate, when combined with platelet protein, they showed partial agglutination.

In the author's experience, the differences between test and controls were often negligible, and, even when present, were not sufficiently convincing to support the continued use of this technique. Further, it has recently been shown that this technique is probably not a measure of a specific anti-platelet factor, but that it would seem to demonstrate a breakdown product of fibrin following fibrinolysis (Dausset et al 1956; Jaeger-Draafsel, Weigman, Fabius & Van Loghem 1956a). This unknown factor was present in large amounts in the blood from cases with cirrhosis of the liver, but is also found in cases of I.T.P. The exact significance of these latter observations is still obscure. Also, it is difficult to define what is present in the platelet protein solution or "extract" that is united with the tanned red cells. This is obtained by breaking up the platelets by repeated freezing and thawing, and after spinning off the platelet debris the supernatant is used as the "platelet extract". Such extracts contain a multiplicity of varying protein coagulation factors (Creveld et al

1951/1952 Ware, Fahey & Seegers 1948; Seegers 1956; Bounameaux 1957a), and it is impossible to ensure that successive extracts are the same and contain the appropriate antigens.

Coombs' Test (Coombs, Mourant & Race 1945).

Neither the direct or indirect Coombs' technique have been used in the present series of experiments as, on preliminary testing, it was found that anti-human-globulin rabbit sera agglutinated all the washed suspensions of human platelets tested.

This result was not unexpected as it was known that platelets carried absorbed globulin as Factor V on their surface (Hjort, Rapaport & Owren 1955). This cannot be removed by repeated washing. Also Bounameaux (1957a) has shown that A.H.G., factor VII, Christmas factor and prothrombin, all known globulins, may adhere to the platelet surface.

Theoretically, therefore, anti-human globulin serum added to human platelet suspensions should provoke agglutination or be neutralised. However, in view of the recent claims by Moulinier (1955), Jaeger-Draafsel et al (1956b) and Nelken & Gurevitch (1956), it would seem that these assumptions may have been erroneous and that this technique should be reinvestigated more fully.

Platelet Agglutination by "Methode d'agitation" of Dausset (Appendix A, p. 20).

Dausset (1954) suggested that, if a mixture of the sera to be tested for the presence of platelet agglutinins was

continuously agitated with the platelets the sensitivity of the agglutination reaction was enhanced. He used platelet-rich plasma as his indicator, and the test serum was exposed to heat at 56°C for 15 minutes prior to testing. A mixture of these was then agitated on a Kline shaker for 30 minutes and the presence or absence of agglutination determined.

During the present investigations, several sera, both normal and from patients with thrombocytopenic purpura, were tested by this method but extremely unsatisfactory results were obtained. Control normal and test sera produced intermittently fibrin clots in the platelet-rich plasma and platelet clumps were formed by both normal and control sera. These clumps appeared morphologically to consist of platelets which had undergone VM.

CHAPTER XV

Discussion

It is impossible to consider the role of platelet agglutination in cases of idiopathic thrombocytopenic purpura (ITP) without some misgiving. Seven years have elapsed since Evans & Duane (1949) opened this new era of research by postulating the existence of an immune mechanism in such cases, and it must be freely admitted that, to date, there is no reliable technique which will give consistent results other than in the hands of its originator. In addition, many earlier claims to have demonstrated anti-platelet factors in ITP cannot be accepted, on grounds of inadequate technique. In spite of Stefanini's and Silverberg's (1951) warning that heat treatment of the test serum (56° for 30 mins.) did not entirely remove the non-specific platelet agglutinating effect of normal serum, several workers, notably Dausset (1954) and Dausset & Malinvaud (1954) ignored this observation and continued to use only heat treated serum (56° for 15 mins.) in their tests. In their original work on this subject, Dausset and his co-workers claimed to have demonstrated positive agglutination tests in ITP using serum which had not even been heat treated. They supported their results by a series of pictures which obviously demonstrated platelet VM and not simple agglutination (see Chapter II) (Dausset et al 1952).

The earlier claims by Kissemeyer-Nielsen (1953a) and others to have detected specific anti-platelet factors in blood by use of the tanned red blood cell-platelet-protein agglutination test have also not been confirmed (Dausset et al 1956).

However, the results from the main American centres of research (Harrington 1954; Stefanini 1955b; Tullis 1956) cannot be criticised on grounds of technique. All these series measured direct agglutination or lysis of platelets. Although Tullis did add serum in a 1/32 dilution to his test system in order to provide Complement, it is unlikely that it had any effect on the platelets when used in such dilutions.

Their results, together with the present series, do demonstrate that in the blood of certain cases of thrombocytopenic and non-thrombocytopenic purpura there exists a factor or factors, which can be shown to be detrimental to platelets in vitro, and which do not appear to be related to any known factor involved in the blood coagulation or Complement systems.

Why this factor is not found in every case is a matter for conjecture. Several opinions have been advanced, all of which are, at present, purely hypothetical. It may be that, in the group of idiopathic purpuras, we are dealing with a protean series of disorders in which only one type is caused by circulating anti-platelet factors. Alternatively, several types of anti-platelet factors may be present, only some of which are demonstrable by direct platelet agglutination

tests. This concept has been supported by the recent work of Jaeger-Draafsel et al (1956b) who claimed that they were able to demonstrate both direct platelet agglutinating factors and incomplete or non-agglutinating factors; the latter being capable of attaching themselves to the platelets without producing their agglutination. A further tenable hypothesis is that cases with negative platelet agglutination tests are those in which all the anti-platelet factors have been consumed during the 'in vitro' platelet destruction.

The techniques may be faulty where normal platelets are used to detect anti-platelet factors. It may be that only the platelets derived from the blood of affected cases carry the appropriate antigen or antigens. The difficulties of isolating platelets from patients with thrombocytopenia tends to preclude the design of a reliable technique to demonstrate this concept in vitro.

The platelet agglutination test, described in this thesis, has been shown, in the study of incompatible combinations of sera and platelets carrying A, B, O, blood group antigens, to be relatively insensitive. It is quite possible that either this technique has detected platelet agglutinating factors only when they have been present in strong concentrations, or the normal platelet suspensions may have been rendered unagglutinable by the process of washing. This latter explanation is a complete antithesis to the observation that platelets, both in whole blood and in washed suspensions, tend to agglutinate at the slightest provocation.

This somewhat critical appraisal of the problem does not include the earlier work by Marino (1905), Ledingham (1914) and Robertson & Lee (1917) and Bedson (1921 & 1922) and others using heterologous anti-platelet sera, nor the work of Ackroyd (1949 a, b, c, 1951, 1952 & 1954) on drug induced purpura. In both examples, platelet agglutination could be demonstrated in vitro with ease, and there was no doubt that agglutination, so produced, was distinct from platelet VM.

The present experimental work has confirmed that human platelets carry the A and B antigens in common with the red cells of the same blood. This has supported the claims of Gurevitch & Nelken (1954, 1955) and Coombs & Bedford (1956) and others and settled the controversy created by Toda (1923), Harrington et al (1953) and Stefanini et al (1953b) who failed to recognise this fact. The claims of the latter workers to have demonstrated platelet groups separate from the red cell groups have received some support from the present experiments performed on normal blood but, as has already been discussed, these results, when analysed, were too ill defined to substantiate the claims of these workers. A strong argument for the existence for separate platelet groups has been presented by those cases of neonatal or congenital thrombocytopenic purpura arising in infants born of normal mothers. While most of these cases apparently occur in children of thrombocytopenic mothers, the former group are being

encountered more frequently, no doubt due to an increasing awareness of the condition, and if platelet counts were done more frequently in cases of haemorrhagic disease of the newborn, it is possible that even more cases would be found. The analogy to ABO and Rhesus incompatibility of the red cells and haemolytic disease of the newborn has been convincing, but in the present series of 5 cases this hypothesis has not been wholly confirmed. In 3 cases, where the mothers had never been transfused or had miscarriages, it was reasonable to accept that the first pregnancies had resulted in affected children. This, together with the ability of the mother's serum in Case 34 to agglutinate in vitro, not only her own platelets, but those of her husband and of normal persons, casts some doubt on the validity of the platelet-group hypothesis. This mother was never found to be thrombocytopenic. The lack of immunising stimulus can be explained if the presence of naturally occurring platelet group antibodies is accepted, as discussed on page 158. Then, if the foetus carried a foreign platelet antigen, the mother's naturally occurring platelet agglutinin might be increased in strength so that it would be capable of destroying the foetal platelets, if trans-placental transfer were to occur at term.

It has not been possible to confirm the antigenic structures detailed in Table XLVI by transfusion studies in cases of thrombocytopenia. Specific platelet transfusions have only been indicated clinically on one occasion, and

in a case with a negative platelet agglutination test. Further, platelet transfusions can be antigenic and their indiscriminate experimental use might well have prejudiced the beneficial effect of a subsequent platelet transfusion required to control intractable haemorrhage. Disappointingly, heterologous anti-human platelet sera did not provide any confirmation of the distribution of platelet group (p.148). Further experimental work with similar heterologous serum and selective cross absorption of sera containing potent platelet agglutinins, by varying platelet suspensions, may confirm the presence of separate platelet groups. At present, the evidence, though suggestive, is extremely tenuous.

If this hypothesis of separate platelet groups is unacceptable, the occurrence of positive agglutination tests in normals, unexplainable in terms of ABO incompatibility between platelets and sera, can only be an expression of the inadequacy of the platelet agglutination technique and must represent false positive results.

Perhaps the strongest support for the existence of anti-platelet factors in thrombocytopenic purpura, lies in the transfusion experiments described by Harrington et al (1951) and Sprague et al (1952). The fact that blood from certain thrombocytopenic patients was capable of inducing thrombocytopenia and even purpura when transfused into normal recipients, strongly supported the idea that these bloods contain anti-platelet antibodies. This property of blood from

cases of ITP can be reproduced by the intravenous administration of fresh serum (Stefanini & Chatterjea 1952), and it would appear that thrombocytopenic blood may differ from normal blood, in that when it is transfused, it behaves as fresh serum and may induce thrombocytopenia by some, as yet undetermined, coagulant activity rather than by any platelet antibody content.

The present series of experiments did demonstrate a platelet agglutinating factor in the sera from certain cases of thrombocytopenic and non-thrombocytopenic purpura and related syndromes. Whatever the nature of this agglutinating factor it is quite certain that its properties are distinct from those of the platelet agglutinating or viscous metamorphosing factors in normal fresh serum. Serum derived from thrombocytopenic blood cannot, however, be considered normal and apart from the large amount of unconsumed prothrombin present (Quick & Favre-Gilly 1949) other residual platelet viscous metamorphosing factors may be present. The aging, heat, and adsorption process, adopted to treat the sera in these cases before testing, ruled out the possibility of existing thrombin, thrombin generated from prothrombin by the addition of platelets to the serum, or any other known serum factor, being the platelet agglutinating factor. The unidentified viscous metamorphosing factor, responsible for the initial

agglutination of platelets in blood coagulation described in Chapters III and IV of this thesis, may not be utilised completely as thrombocytopenic blood clots. It has been impossible to discover whether this factor is neutralised by heat, or barium sulphate absorption, in the native plasma system used to demonstrate its existence. Therefore it cannot be ruled out that unconsumed amounts of this factor may be present in serum derived from cases of thrombocytopenia and, unaffected by heat or barium sulphate absorption, be capable of producing platelet agglutination in the test mixtures. If, however, the positive tests were due to unconsumed amounts of this or any other VM factor, they should have been obtained with every serum derived from thrombocytopenic blood. This was certainly not so, and the sera which gave positive results, produced platelet agglutination and not, if I interpret the change correctly, VM.

It has been assumed by nearly every worker that they were measuring a specific platelet antibody in the form of an agglutinin or lysin and those who have considered alternatives have also concluded that this was so (Stefanini et al 1953a; Tullis 1956). Both these workers based their conclusions on the observation that the factor they considered to be a platelet agglutinin or lysin was adsorbed specifically by platelets and not by red cells and was present in the globulin component of the blood proteins. But, as serum platelet VM factor is absorbed specifically by platelets and is related

to the globulin fraction, albeit different from that containing the platelet agglutinating factor (see Tables XIV and LIV), these criteria would appear to be insufficient evidence upon which to decide that the platelet agglutinating factor in thrombocytopenic blood is a specific platelet antibody.

Although other techniques of established value in serology have been applied to this problem, they have been less reliable than direct platelet agglutination techniques. Coombs, Marks & Bedford (1956), using a modification of their original mixed erythrocyte-platelet agglutination technique were not able to demonstrate positive results in thrombocytopenic purpura while strong positive results were obtained with potent anti-human platelet rabbit sera. They suggested tentatively that perhaps the platelet agglutinating factor found in certain cases of thrombocytopenic purpura was not an antibody. This, as they state, is a serious conclusion in view of all the published results on this subject. Yet the so many conflicting results obtained in the search for these so-called antibodies, and the protean nature of the latter, tend to suggest that this dissenting opinion may be sustained. If not an antibody or a coagulation factor, some other explanation must be offered for the presence of these platelet agglutinating factors. These must be largely hypothetical, but nevertheless of value.

It has been daimed that alteration in the platelet surroundings may induce non-specific platelet agglutination by disturbing their equilibrium (Achard & Aynaud 1908 a, b, c, and

Tocantins 1938). They cited a wide variety of agents capable of this action: egg albumin; gelatin, gum arabic, egg lecithin, collokdal arsenic and peptone, and Achard & Aynaud (1908c) drew attention to their common colloidal nature. Starlinger & Sametnick (1927) considered that platelets could be agglutinated by any increase in the negatively charged proteins (fibrinogen and globulin fractions). These, by lowering the electric charge of the platelets, themselves negatively charged (Starlinger et al 1927; Wright 1951; Bigelow & Desforges 1953) allowed them to approach more closely to one another and agglutinate. This action Wright (1951) considered, was due to these proteins being hydrophilic colloids, capable of reducing surface charge by modifying the position adsorbed water on the cell surface. It is possible that such non-specific alterationsoof the blood might induce platelet agglutination in vivo or in vitro and could explain some of the observed phenomena.

Within the past 2 years two original communications from Switzerland have suggested yet a further explanation. Hoigne, Grossman & Stork (1955) have described how they were able to demonstrate a fall in the platelet count in patients in whom they provoked an antigen-antibody reaction. They further claimed that they could also demonstrate this phenomenon in vitro with associated platelet agglutination. That such a mechanism exists has been demonstrated experimentally in rabbits (Miescher & Straessle 1956). By using radioactive tracer techniques these workers were able to demonstrate that

soluble antigen-antibody complexes in the blood stream become firmly fixed to the platelets. In one instance strong platelet agglutination was provoked when such a reaction was induced experimentally. The factors controlling the presence of agglutination appear to be, the nature of the antigen, the antibody, and the ratio of one to the other. This latter factor was also stressed by Hoigné et al (1955) who found that platelet agglutination only occurred in vitro when an optimum concentration of antigen has been achieved and this lay within a very narrow range. These observations are important and the critical ratio of antigen-antibody may explain some of the observed variation in experimental work in cases of thrombocytopenia. It is highly probable that such a mechanism existed in Case 65 where apparent sensitivity to human albumen could be demonstrated by in vitro platelet agglutination. Certain features of this hypothesis are similar to these suggested by Ackroyd (1955a) to explain the mechanism in drug sensitisation purpura. For this very reason, further experimental work on similar lines to Hoigne et al (1955) and Miescher et al (1956) may be fruitful. If such a mechanism does exist in ITP it is probable that in vitro platelet agglutination tests may be a measure of free unbound antigen antibody complex. If, however, the soluble antigen-antibody complex has been bound completely to the platelets, while provoking agglutination 'in vivo', no platelet agglutination phenomenon would be demonstrable in 'vitro'.

This might explain the negative results obtained in this and other series, and it would be unlikely that such a mechanism could be demonstrated other than by direct platelet agglutination tests or by the use of anti-human globulin serum (Coombs et al 1945).

If either of these hypotheses (i.e. abnormal amounts of normal proteins or an antigen-antibody complex) be accepted, even tentatively, as an explanation of the pathogenesis of ITP, some explanation must be offered for their presence. Stefanini (1955a) has suggested that viruses, bacteria, or drugs may alter normal protein synthesis in such a way that the abnormal or abnormal amounts of certain protein fractions may be produced which will behave as "antibodies". A gross example of such a 'dysproteinaemia' is illustrated by the three cases with cryoglobulinaemia (Cases 52, 53 & 54) encountered in the present series and may be by the one case of virus pneumonia (Case 72). An antigen-antibody reaction might result from action of viruses, bacteria or drugs on the platelets, bestowing antigenic properties on these cells by altering their structure, or, akin to Ackroyd's hypothesis conferring antigenic powers on the platelet by acting as haptens. The resultant antibodies would, when formed, react with the appropriate antigen, damage the platelets, and so induce thrombocytopenia. If this is so, in vitro tests might only give reliable results when the patient's platelets and not normal platelets are used as the 'antigenic' material. This concept has received experimental support (Kistner &

Stefanini 1956). These workers have claimed that they were able to modify rabbit platelet suspensions in vitro, with drugs, bacteria, and viruses, so that when the former were re-injected into the donor rabbit, it appeared to become immunised against its own modified platelets (auto-immunisation) in some instances.

Alternatively viruses, bacteria, drugs or proteins, antigenic in their own right, may attach themselves to the platelets and, on reacting with their own specific antibodies, provoke platelet agglutination and even lysis both 'in vivo' and 'in vitro.'

That bacteria or other particulate matter may unite with the platelets has been claimed by Aynaud (1911b), Tocantins (1938) and Bloom, Gustavsen & Swenson (1955). Aynaud (1911b) claimed that bacteria by themselves, directly agglutinated platelets in dog and rabbit blood. This, he claimed, was by virtue of their altering the physico-chemical equilibrium of the blood. The factors involved in maintaining this physico-chemical equilibrium have still to be defined.

There is abundant evidence that virus diseases are often accompanied by thrombocytopenia (Hayem & Bensaude 1901 - smallpox; Achroyd 1949d- rubella; Motulsky 1953 - influenza; Jorgensen 1953 - glandular fever; Belber, Davies & Epstein 1954 - cat scratch disease; Moeschlin, Siegenthaler, Gasser & Hassig 1954 - virus pneumonia; Hudson, Weinstein & Chang 1956 - measles) and indefinite febrile illnesses are quite frequently encountered immediately prior to the onset of

thrombocytopenic purpura (Robson 1954).

If it be accepted that anti-platelet agglutinins or lysins exist in the blood of thrombocytopenic and certain non-thrombocytopenic purpuras, it is necessary to consider how they produce thrombocytopenia or purpura. They may induce thrombocytopenia by destruction of the circulating platelets in the blood by either lysing the platelets or by altering them in such a way that they are phagocytosed by circulating monocytes (Bessis & Tabuis 1954) or by the reticulo-endothelial system (Bedson 1926). Alternatively it has been suggested by Tocantins & Stewart (1939) and Pisciotta et al (1953) that they may act directly on the megakaryocytes, preventing the normal maturation of the cells and platelet production. Their action could, of course, be a summation of these two actions.

The role of the spleen is still controversial. It is assumed by many that by virtue of its content of reticulo-endothelial cells it will destroy the platelets damaged by circulating platelet antibodies and thus when the spleen is removed the platelets may return to normal levels. Yet other organs contain reticulo-endothelial elements and these should be capable of taking over the phagocytic action of the spleen. In those cases in the present series where the platelet agglutination test was performed after successful splenectomy, the agglutinin had either disappeared or the titre had fallen. This suggested that the role of the spleen in

thrombocytopenic purpura might well be the production of the platelet agglutinating factor with or without concomitant platelet destruction in the organ. This view, of course, reverts to the original hypothesis of Troland & Lee (1938). It received some support from the observation of Stefanini (1955b) who claimed to have isolated platelet agglutinins from the spleen of cases where agglutinins were detected in the blood. As he admits, however, this finding might represent merely a saturation of the tissues by the agglutinating factors.

As has already been discussed in the introduction to Part I of this thesis, thrombocytopenia per se, does not explain the occurrence of purpura and other spontaneous haemorrhages in these cases and it is attractive to speculate whether the anti-platelet factors might have some simultaneous action on the blood vessels themselves. This they might do, as Hayem (1896) suggested, by producing blockage and infarction of small vessels by the agglutinated platelet masses. Alternatively, if Clark & Jacob's (1950) hypothesis is sustained, it is possible that vascular endothelium, sharing a common antigen with the platelets, may be damaged as the thrombocytopenia is induced.

This discussion of the nature of the platelet agglutinating factor and the pathogenesis of ITP has been rich in speculation, but it has served to show that this subject is far from being decided as the modern literature has tended to suggest and it has provided several important hypotheses

upon which future research may be based.

Whatever the explanation of the pathogenesis of the idiopathic thrombocytopenic syndrome, it is possible that all these mechanisms may be involved and if so, it is futile to expect one standard technique to demonstrate all these factors. It may well be necessary to use a battery of investigations in order to determine the pathogenesis of any one case of thrombocytopenic purpura.

Lastly it is necessary to ascertain whether the knowledge gleaned from this, and other series of observations, has provided the clinician with anything other than a rather ill-defined academic explanation for the pathogenesis of certain cases of thrombocytopenic and non-thrombocytopenic purpura.

The present series describes the results obtained using one particular test to examine the blood from 73 cases of purpura, and related syndromes, encountered in general hospital practice in a period of two years. The technique used, while not difficult, required careful control and is not yet suitable for use as a routine test. To prepare, on every occasion, satisfactory suspensions of platelets, siliconed glassware, and large and multiple specimens of normal blood, is time-consuming, and not every laboratory staff is willing to provide the multiple specimens of blood required. It must be stressed that the use of control samples from so-called normal patients, may be misleading. In addition, as in so

many other laboratory tests, it is difficult to maintain a satisfactory standard and adequate controls. Unless the technique is being used at frequent intervals, it is doubtful whether the results obtained can provide information that may modify clinical judgement of a given case. One exception is in those cases of possible drug purpura where a platelet agglutination test may, together with other tests, pinpoint the offending drug (Case 35). The general trend towards a higher number of positive tests in chronic thrombocytopenic purpura does suggest that a positive result obtained in the early stages of the disease may indicate that a spontaneous remission will not occur. The present series has not confirmed that cases with a positive agglutination test have a more favourable response to splenectomy than those with negative results, nor do the results suggest that any forecast can be made in respect of the response to steroid therapy. Thus, clinical judgement must still be the criteria for the selection of therapy in cases of thrombocytopenia of whatever cause.

A positive result in any given case would, on theoretical grounds, suggest that platelet or fresh blood transfusions would be valueless as immediate destruction of the transfused platelets should take place. Again, however, it would not

appear to be justified to withhold such therapy because of a positive agglutination test in the recipient. Platelet transfusions are usually reserved for the control of intractable spontaneous haemorrhage which is endangering life and, in such instances, may improve haemostatic function without any apparent survival of transfused platelets. Further platelet transfusion may provoke a spontaneous remission in the thrombocytopenic recipient (Stefanini, Chatterjea, Dameshek, Zannos & Santiago 1952; Creveld, Paulssen & Bartels 1953).

In neonatal thrombocytopenia encountered in children of normal mothers, the finding of a platelet agglutinating factor in the mother's serum may provide evidence upon which to base prognosis both in relation to the existing case and to future children. A negative finding would suggest that some other factor such as infection or congenital marrow aplasia might be present (Stefanini & Dameshek 1955). It is, however, by no means certain that it is correct, on the basis of this test, to give an unfavourable prognosis in regard to the incidence of thrombocytopenic purpura in subsequent children. Such a prognosis was given in one case in the present series, and the subsequent child was, as predicted, thrombocytopenic. However, the fallacies of the test are such that this advice might have been misleading. Conversely, a positive result such as obtained in Cases 30 and 34 does indicate that subsequent pregnancies should be carefully

observed and delivery performed in a hospital centre equipped for providing fresh platelet transfusion.

Therefore, except in these quoted instances, the platelet agglutination test does not appear to help the clinician towards a differential diagnosis in cases of thrombocytopenic or non-thrombocytopenic purpura, nor does it aid him in his decision as to the therapy or the prognosis of a given case.

TABLE LV

Abbreviations

Spleen +	=	Enlarged
Spleen -	=	Normal size
B.T.	=	Blood Transfusion
Preg.	=	Pregnancy
I.T.P.	=	Idiopathic Thrombocytopenic Purpura
T.P.	=	Thrombocytopenic Purpura
N.T.P.	=	Non-thrombocytopenic Purpura
A.D.L.E.	=	Acute Disseminated Lupus Erythematosus
R.	=	Response to therapy
N.R.	=	No response to therapy
P.R.	=	Partial response to therapy
T.R.	=	Transient response to therapy

1	F	57	Acute I.T.P.	2 mos.	10,000			-	+	-	-	+ H.R.	-	
2	F	22	Recurrent Chronic I.T.P.	6 years	19,100	+ - (Post Splen-ectomy)	-	+	+	+	+	+ R.	+ R.	
3	M	14	Recurrent Chronic I.T.P.	11 yrs.	10 - 30,000	+	1/8	-	+	-	-	-	-	Splenectomy refused
4	F	28	Acute I.T.P.	5 days	Too few to cout	-		-	+	-	-	+ R.	-	
5	F	52	Recurrent Chronic I.T.P.	3 years	40,000	+	-	+	Not rec.	-	+	-	-	Therapy refused
6	M	30	Acute I.T.P.	2 days	Too few to cxunt	-		-	+	-	-	+ R.	-	
7	F	60	Chronic I.T.P.	8 mos.	9,000	+	1/4	-	+	-	+	-	-	Splenectomy advised
8	F	59	Chronic I.T.P.	6 mos.	7,000	+ (+ (Post Splen-ectomy)	1/32 1/16	+	+	+	+	+ P.R	+ R.	Radium menopause

9	M	52	Chronic I.T.P.	2 years	38,000		−		+	Not recor.	+	−	−	+ R.	Radiotherapy 1954. Hypoplastic marrow 1955. Relapse 10/12 after splenectomy
10	M	35	Chronic I.T.P.	9 mos.	10 − 60,000		+	¼	+	+	+	−	−	−	
11	F	24	Recurrent Chronic I.T.P.	16 yrs.	14,000		+	¼	−	+	−	−	−	−	Splenectomy advised
12	F	10	Chronic I.T.P.	2 years	Too few to count		−		−	Not recor.	−	−	−	+ R.	
13	F	61	Recurrent Chronic I.T.P.	12 yrs.	12,000		−		−	+	+	+	+ R.	+ R.	Relapse 9 years after splenectomy
14	M	10	Acute I.T.P.	1 week	Too few to count		+	½	−	Not recor.	−	−	−	+ R.	
							+ (Post Splen)	½							
15	F	70	Chronic I.T.P.	1 year	Too few to count		−		−	−	+	+	+ N.R.	+ P.R.	
16	M	9	Acute I.T.P.	2 mos.	10,000		−		+	Not recor.	−	−	+ N.R.	−	Spontaneous remission

17	M	10	Acute I.T.P.	2 mos.	36 – 60,000	–		+	+	+	–	+ N.R.	+ N.R.	
18	M	3½	Acute I.T.P.	5 days	10,000	–		–	Not recor.	–	–	–	–	Spontaneous remission
19	M	48	Chronic I.T.P.	2 years	Too few to count	+	⅛	–	+	–	–	+ N.R.	+	Died following splenectomy
20	F	31	Chronic T.P.	20 yrs.	32,000	–		–	Not recor.	+	+	–	–	Probable hereditary platelet defect
21	F	75	I.T.P.	Unknown	50,000	–		–	Not recor.	Unknown	+	–	–	
22	F	26	Chronic I.T.P.	7 years	21,000	–		–	–	+	+	+ P.R.	+ N.R.	
23	F	42	I.T.	Unknown	109,000	+	½	–	Not recor.	–	+	–	–	
24	F	51	Acute I.T.P.	Several weeks	9,000	–		–	+	–	+	+ R.	–	
25	M	13	Chronic I.T.P.& Pancyto-penia	3 years	15,000	+ – (Post Splen.	⅛	+	+	+	–	+ P.R.	+ P.R.	Clinically well in spite of persis-tent thrombocytopen ia Associated with *anaemia & leucopenia*

			Recurrent													
26	F	6	Chronic I.T.P.	3 years	42,000		+	1/8	-	Not recor.	+	-	+ N.R.	+ N.R.		
27	M	18	Chronic I.T.P.	6 mos. +	41,000		+ + (Post Splen.)	1 1/2	-	+	-	-	-	+ R.		
28	M	50	Acute I.T.P.	5 days	54,000		-		-	Not recor.	-	-	-	-		Spontaneous remission
28a	F	57	Recurrent Chronic I.T.P.	20 yrs.	40,000		-		-	+	-	+	+ P.R.	-		
29	F	39	I.T.P.	Unknown	72,000		-		-	Not recor.	+	-	-	-		Associated with Ehlers Danlos Syndrome
30	F	27	-	-	212,000	-	+	1/2	-	-	-	+	-	-		Mother never had any haemorrhagic manifestations
	M	5 d.	Neonatal T.P.	10 days (died)	Too few to count				Not recor.	Not recor.	-	-	-	-		
31	F	24	-	-	406,000	-	+	1/1	-	Not recor.	-	-	-	-		1st pregnancy
	M	5d.	Neonatal T.P.	4 days. Persisted for 6 weeks	10,000		-		-	Not recor.						

32	F	25	–	–	454,000	–	+	1/16	–	Not exam.	–	+	–	–	Previous child (1st pregnancy) als affected
	M	2d.	T.P.	2 days persisted	Too few to count for 8 wks		–		–	Not exam.	–	–	–	–	
33	F	28	–	–	336,000	–	–		–	Not exam.	–	+	–	–	
	M	5d.	Neonatal T.P.	1 day persisted for 1 week	62,000	–	–		–	Not exam.	–	–	–	–	
34	F	24	–	–	132-260,000	+	+	¼	+	Not exam.	–	+	–	–	First child male also affected
	M^1	2d.	Neonatal T.P.	8 days (died)	15,000				Unknown	Not exam.	–	–	–	–	P.M. Extensive purpura
	M^2	28d	Neonatal T.P.	Persisted 6 weeks	15,000	–	+		–	Not exam.	–	–	–	–	Now entirely normal child
35	F	80	Drug T.P.	1 week	129,000	+	+	1/1	–	Not exam.	–	+	–	–	Sensitivity to Histantin
36	M	72	Drug T.P.	10 days	80,000	+	+	¼	–	Not exam.	–	–	–	–	Sensitivity to Sulphamezathine

37	F	24	Drug T.P.	Unknown	33,000		-		-	-	-	+	-	-	Associated agranulocytosis due to Thiouracil therapy
38	F	56	Toxic T.P.	1 month	53,000		-			Not exam.	-	-	-	-	Sensitivity to floor polish
39	F	59	Hyper-splenism	5 mos.	25,000	{	+ / - (post op.)	½	+	-	+	+	-	-	Associated with splenomegaly & cirhosis of the liver. Remission following porto-caval anastomosis
40	F	3	Secon-dary T.P.	6 mos.	15,000		-		+	-	+	-	+ N.R.	-	Acute Leukaemia
41	M	57	Secon-dary T.P.	1 week	15,000		-		-	-	-	-	-	-	Disseminated carcinomatosis
42	F	56	Secon-dary T.P.	6 mos.	16,000		-		+	-	+	+	+ N.R.	-	Histiocytic medullary reticulo-sis
43	F	17	Secon-dary T.P.	6 mos.	12,000		-		+	+	-	-	+ T.R.	-	Histocytic medullary reticu-losis or acute Hodgkins disease

44	F	49	Secondary T.P.	3 yrs.	50–70,000	–		+	±	+	+	–	–	Disseminated carcinomatosis
45	M	53	Secondary T.P.	5 weeks	19,000	–		–	–	–	–	+ N.R.	–	Acute myeloid leukaemia
46	F	57	Secondary T.P.	Unknown	95,000	+	1/1	+	Not exam.	–	+	–	–	Disseminated carcinomatosis
47	M	67	T.P.	Unknown	17,000	–		+	Not exam.	Unknown	–	–	–	Associated with congestive cardiac failure
48	F	40	T.P.	Unknown	18,000	–		–	Not exam.	–	+	–	–	Defibrination syndrome of pregnancy
49	F	28	T.P.	10 days	Too few to count	–		–	Not exam.	+	+	–	–	Defibrination syndrome of pregnancy
50	M	67	I.T.P.	1 week	92,000	–		+	+	–	–	+ R.	–	Associated with haemolytic anaemia
51	F	34	I.T.P.	2 yrs.	45,000	+ / – after steroid therapy	1/4	+	Not exam.	+	+	N.R. Cortisone +R. Prednisolone	+T.R.	Associated with haemolytic anaemia

52	F	56	T.P.	2 mos.	89,000	+	+	-	-	+	-	+	+ N.R.	-	Associated with cryoglobulinaemia
53	M	49	T.P.	3 yrs.	84,000	+	+	-	+	+	-	-	-	-	Associated with cryoglobulinaemia
54	M	59	N.T.P.	9 yrs.	234,000	+	+	-	-	+	-	-	+ N.R.	-	Associated with cryoglobulinaemia
55	M	56	ADLE	3 yrs	254,000	-	-		+	Not exam.	-	-	+R	-	L.E. cells present in large numbers
56	M	59	ADLE	Unknown	33,000	-	-		+	Not exam.	-	-	+	-	Scanty L.E. cells present
57	F	54	N.T.P.	3 mos.	229,000	-	-		-	Not exam.	-	-	-	-	Associated with progressive lower motor neurone disease
58	M	70	N.T.P.	18 yrs.	309,000	+	+	1/1	-	Not exam.	+ (100+)	-	+ N.R.	-	Associated with abnormally low levels of platelet *5-hydroxytryptamine*
59	F	26	N.T.P.	2 mos.	197,000	-	-		-	Not exam.	-	+	+ R.	-	Henoch Schonlein type purpura

60	M	43	N.T.P.	3 wks.	205,000		-		-	+	-	-	+ N.R.	-	Henoch Schonlein type purpura
61	M	65	N.T.P.	1 week	321,000	-	-		-	Not exam.	-	-	-	-	Henoch Schonlein type purpura
62	F	77	N.T.P.	Several days	190-262,000	-	-		-	Not exam.	-	+	-	-	Henoch Schonlein type purpura
63	M	59	N.T.P.	2 yrs.	215,000	-	-		-	Not exam.	+	-	-	-	
64	F	24	N.T.P.	2 days	239,000	+	+	½	-	Not exam.	+	+	-	-	Associated ulcerative colitis
65	M	15	N.T.P.	1 day	207,000	+	+		-	Not exam.	+	-	-	-	Sensitivity to human Albumen (Lister)
66	F	49	N.T.P.	1 week	217,000	-	-		-	Not exam.	-	-	-	-	
67	F	17	N.T.P.	6 mos.	206,000	-	-		-	Not exam.	+	-	-	-	Unexplained failure of skin grafting operations
68	F	5	Intra-ocular haemorrhage	Unknown	216,000	-	-		-	Not exam.	-	-	-	-	

TABLE LV (See also Appendix C)

Case No.	Sex	Age	Diagnosis	Duration	Platelet Count /cu.mm.	Platelet Agglutination Test			Spleen	Mega.	Previous B.T.	Previous Preg.	Steroid Therapy	Splen-ectomy	Remarks
						Auto Agglutinins	Iso Agglutinins	Titre							
69	F	38	N.T.P.	3 years	312,000	-	-		-	Not exam.	-	+	-	-	
70	F	51	N.T.P.	2 mos.	189,000	-	-		-	Not exam.	-	-	-	-	
71	F	46	N.T.P.	2 mos.	362,000	-	-		-	Not exam.	-	-	-	-	Spontaneous remission
72	M	30	Virus Pneumonia	1 month	312,000	+	+		-	Not exam.	-	-	-	-	

CHAPTER XVI

In Conclusion

The study of immune reactions involving blood platelets has been complicated by the ability of these cells to undergo clumping and lysis during the normal physiological process of blood coagulation. This change is similar to the sequence of agglutination and lysis which cells undergo when acted upon by specific immune antibodies and therefore it was of paramount importance to separate these two phenomena. Many workers have tried to circumvent this problem by evolving techniques to detect anti-platelet immune antibodies that did not necessitate the measurement of platelet agglutination. These, while apparently giving reliable results in the hands of their originators, have tended to be unreliable in the hands of other workers. Platelet agglutination techniques seem to provide more reproducible results.

The factor or factors that control physiological platelet agglutination during the sequence of blood coagulation have been the subject of controversy, and a fuller understanding of these factors had to be reached before any analysis of the results of other workers could be made, or a suitable technique evolved, for measuring immune platelet agglutination. The experimental results detailed in Part 1 of this thesis have advanced our understanding of physiological viscous metamorphosis (VM). The latter would appear to be an

important feature of blood coagulation as it is the first visible change that takes place in shed blood and precedes fibrin formation by approximately two minutes. During VM the platelets would appear to release granular material which is thought to supply their contribution to intrinsic plasma thromboplastin generation.

It is the belief of many workers that thrombin, and thrombin alone, is always responsible for VM. Purified human thrombin has been shown in Chapter V to be capable of producing VM in the presence of a labile co-factor attached to the surface of the platelets. Bounameaux (1957 a & b) and Luscher (1957) have evolved an attractive hypothesis, based on the observed concentration of coagulation factors on the surface of the platelets. They believe that coagulation starts on the surface of the platelet, as in whole blood, and that trace amounts of thrombin are generated rapidly on this surface which will induce the full sequence of VM in platelets long before fibrin appears. If this is true, in severe haemophilia and Christmas disease, the platelets are probably as deficient in antihaemophilic globulin or Christmas factor as the surrounding plasma. Therefore it is unlikely that thrombin could be generated rapidly on their platelet surfaces. However, during the experiments performed on platelet-rich native plasma obtained from severely affected cases of haemophilia and Christmas disease, VM took place within minutes of the blood commencing to clot. In addition, heparin, a potent anti-thrombin, also failed to inhibit this early VM

except when used in high concentrations. Thus the Bounameaux-Luscher hypothesis cannot be sustained.

This property of thrombin is dependent on the assumption that the human thrombin used in these experiments was functionally pure. It is by no means certain that this was so, for it cannot be ruled out that this thrombin does not contain other factors which might be capable of producing VM independently from the conversion of fibrinogen to fibrin by thrombin itself. The factor in fresh serum, which will induce VM in washed platelet suspensions, has still to be identified, but it was not shown to be thrombin. It could be the same as the intermediate product of thromboplastin formation, as suggested by Bergsagel (1956), and this latter factor could also be present as a contaminant in the 'pure' human thrombin used in these experiments.

Bergsagel (1956) considered that VM was induced by an intermediate product of thromboplastin formation which was the result of an interaction between antihaemophilic globulin and Christmas factor and calcium. He did not think thrombin had any action in the platelets. If this were correct, VM should not take place in clotting platelet-rich plasma derived from cases of haemophilia or Christmas disease. The present experiments have shown clearly that this was not so, and therefore Bergsagel's hypothesis could not be confirmed.

Repetition of Bergsagel's experiments has suggested that the VM factor in his mixtures was thrombin, but it cannot be

excluded that the thrombin converted fibrinogen to fibrin, incidental to the VM, and that Bergsagel's observations were the result of yet another, as yet undetermined, VM factor, similar or identical to that responsible for the production of VM by serum or in the initial stages of blood coagulation.

This study of VM, and the factors which control its evolution, have tended to complicate rather than simplify the problem of differentiating immune platelet agglutinins from those involved in the blood coagulation process, but it has been possible to demonstrate that the morphological appearances of VM are different from those of immune platelet agglutination. From the experimental data obtained during this study of the behaviour of platelets in blood coagulation, it was possible to decide upon a technique to measure platelet agglutinating factors, which excluded all known coagulation and non-specific factors which might produce platelet clumping. Whether other VM factors might exist which, although not present in normal serum, might be found in serum derived from thrombocytopenic blood, but if this were so, all sera prepared from thrombocytopenic blood should be capable of inducing platelet clumping after appropriate treatment to exclude known VM factors. This was not found to be the case.

In order to establish whether this theoretically optimum platelet agglutination test was of practical use, a series of tests using normal sera was performed. Platelet suspensions were prepared from the same normal donors as the sera, and, by cross agglutinating these sera with the platelet suspensions in groups of ten, it was possible to observe whether normal

sera could agglutinate normal platelet suspensions. This exercise provided a great deal of useful information, as a surprisingly high number of positive results were obtained. Certain platelet suspensions were more readily agglutinated than others, and this suggested that some group structure existed on the platelets.

Using specific anti-A and B sera, it was confirmed that A and B antigens of the red blood cells were shared by the platelets. Yet only very high titre sera were capable of provoking direct agglutination and not all platelet suspensions from group A and B donors could be so agglutinated. This failure to agglutinate appeared to be confined to those platelet suspensions from group A and B donors who were non-secretors of A and B substance. This led to the conclusion that the A and B antigenicity of platelets might be due to A and B substance from the plasma on to the platelet surface, but this hypothesis was not confirmed by the mixed red cell-platelet agglutination technique devised by Coombs & Bedford (1955). This showed clearly that the non-secretors, as well as secretors of A and B substance, carried the A and B antigens on their platelets. After excluding those positive agglutination tests which could be explained in terms of ABO incompatibility, a significant number of positive results still remained. The Rhesus antigen D and the Forssman antigen were not found to be present in the platelets and there was no evidence that any other known red cell antigen was shared by

the platelets. Therefore it was considered that these remaining positive results might represent a separate platelet group system, or were simply false positive results. A theoretical platelet group structure was composed from these cross agglutination experiments, but it has not been possible to confirm this serologically or by transfusion studies.

When sera were prepared from patients with thrombocytopenic and non-thrombocytopenic purpura and related conditions, and tested by the platelet agglutination test, a much higher incidence of positive results was obtained than when normal sera were used. The distribution of the results was somewhat haphazard, but there was a higher incidence of positive results in those sera from cases of long standing thrombocytopenic purpura and from the mothers of infants with neonatal thrombocytopenia than in other groups. These results were not thought to be a measure of a physiological VM factor, as morphologically this platelet agglutination was similar to that produced by immune sera. Therefore it was possible that these positive agglutination reactions could be a measure of immune platelet agglutinins and so a confirmation of the hypothesis of immune mechanisms in the pathogenesis of thrombocytopenic purpura.

Why positive results should not be found in all cases of idiopathic thrombocytopenia and why certain other techniques have failed to give reproducible results, remains a mystery. Various explanations have been discussed in the previous chapter and it must be concluded that more experimental work must be performed before these problems can be answered.

Disappointingly, an analysis of positive results obtained in these cases did not appear to offer any advance in our knowledge of the pathogenesis of these conditions, nor provide any guide as to appropriate therapy. The platelet agglutination test may assist in assessing the prognosis of an individual case and this is especially true for cases of neonatal thrombocytopenic purpura.

To summarise, this thesis has established:-

(1) that two types of platelet agglutination exist, namely physiological agglutination or VM, and immune agglutination, and that these may be differentiated morphologically;

(2) that VM of platelets is an essential feature of the sequence of blood coagulation and that during this process the platelets release material essential for thromboplastin formation;

(3) that while thrombin does appear to be capable of promoting typical VM, it is not the only factor that can do so. At least two others must exist, one in the initial stages of the sequence of blood coagulation requiring the presence of Rosenthal's factor (P.T.A.), the other in fresh serum;

(4) that it is possible to destroy or neutralise all the VM properties in a given serum. Therefore, if this serum will then produce platelet clumping, the latter must represent some process, other than VM;

(5) that platelets do carry the ABO antigens according to the red cell group of the original blood;

(6) that a theoretical platelet group structure can be devised;

(7) that sera derived from certain cases of idiopathic thrombocytopenic purpura do contain platelet agglutinating factors, but it has still to be demonstrated beyond all possible doubt that these are specific platelet immune antibodies. Alternative explanations for the existence of these factors have been discussed.

Therefore this thesis has established its original purpose, namely to study viscous metamorphosis and platelet agglutination in order that the techniques of other workers could be analysed and a test devised to measure platelet agglutinating factors, other than those concerned in the sequence of blood coagulation, in normal and abnormal blood.

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APPENDIX A

REAGENTS AND TECHNICAL METHODS

Glass Washing

All glassware used in the experimental work described in this thesis was cleaned as follows:

Tubes and pipettes were given a preliminary wash using warm tap water containing a few drops of detergent ('Teepol' - Shell) to remove any residual plasma, serum, whole blood, or other proteinaceous material.

Following this preliminary wash, the glassware was rinsed in clean tap water and then stored prior to bulk washing. Bulk washing of tubes and glass containers, other than pipettes, was carried out by boiling the glassware in distilled water containing a small amount of 'Brylyanz' (George T. Gurr) for 15 minutes. The glass was then washed thoroughly in N/1000 hydrochloric acid and rinsed thoroughly in several changes of tap water. Final rinsing was performed in distilled water and the glassware dried at 37°C.

Pipettes were washed in several changes of tap water and distilled water by suction. A final rinse with acetone eradicated all traces of moisture.

When glassware was unusually dirty it was immersed for at least 24 hours in concentrated chromic acid (3.6 ml. saturated sodium dichromate in 96.4 ml. concentrated sulphuric acid).

Preparation of Siliconed Glassware

Clean glass surfaces were siliconed by the application of a varnish silicone, MS. 1107 (Midland Silicones Ltd.) diluted to 5% in carbon tetrachloride.

The glass containers or pipettes to be siliconed were filled completely with diluted silicone. The silicone was then discarded into its original container and excess silicone allowed to drain onto filter paper. The siliconed glassware was first dried at room temperature and then baked in a hot air oven for 1 hour at 100°C. This process produced a perfect non-water wettable surface.

All glassware was resiliconed each time it was used.

The siliconed glassware must be handled separately from ordinary glassware used for coagulation or biochemical purposes. This is due to the property of silicones to spread and coat all surfaces such as sinks, washing brushes and other glassware. This was found to be true for the present type of varnished silicone. One sink, test tube brushes and rinsing buckets should be reserved for siliconed glassware only.

Anticoagulants

Trisodium Citrate (Analar) 3.8% w/v.

9 ml. of venous blood was added to 1 ml. sodium citrate and mixed thoroughly.

Sodium Oxalate (Analar) 1.34% w/v.

9 ml. venous blood was added to 1 ml. sodium oxalate and mixed thoroughly.

Disodium Versene ('Sequestrene'; Disodium-ethylene diamine tetra-acetic acid). (J. Light & Co., Colnbrook, Bucks). 4.5% w/v isotonic.

9.7 ml. venous blood was added to 0.3 ml. disodium versene and mixed thoroughly.

Heparin (Boots Ltd.)

Ampoule 5,000 u/ml.

This was diluted in normal saline to obtain the concentrations defined in experiments (Appendix B).

Thrombodym (Neodymnium 3-sulpho-iso nicotinate).

2.5% solution in ampoules. Auer Laboratories, Berlin.

Dilutions were made in isotonic saline.

Soya Bean Trypsin Inhibitor (Pure Crystalline; Kunitz 1945) was provided for experimental use by Dr. R.G. Macfarlane.

Calcium Chloride (Analar)

Molar $CaCl_2$ contains 1 gramme molecular weight or 111 gms/litre.

Anhydrous $CaCl_2$ was dissolved in water to give solutions of strengths M/10, M/20, M/40. As only approximately 65% of this anhydrous salt goes into solution, the solution was made up to give more than the desired strength and the Cl- content titrated with standard silver nitrate. From the calculated concentration of $CaCl_2$ appropriate dilutions were made to give the exact Molar strength required.

Barium Sulphate

X-ray barium sulphate was washed prior to use as a plasma or serum absorbant. Failure to do this resulted in a fine opalescence persisting in the serum or plasma following absorption which in turn led to widespread inaccuracies in subsequent experiments.

1 lb. x-ray barium sulphate was therefore added to 2 litres 0.005M trisodium citrate and the mixture stirred thoroughly. After being allowed to sediment for 5 hours at room temperature, the opalescent supernatant was removed and the sedimented barium sulphate resuspended in a further 2 litres of 0.005M sodium citrate. After 5 hours the opalescent supernatant was removed and the residual sediment filtered through coarse filter paper (Whatman No. 3). The precipitate was dried in the hot air oven for 8 hours at 100°C, ground into a fine powder in a pestle and mortar, redried, reground, and stored for use in a glass bottle.

For optimum absorption of prothrombin, Christmas factor and factor VII, this washed powder was used in the proportion of 200 mgms. barium sulphate/milli-litre of oxalate plasma or serum. (Barium sulphate will not absorb these factors from citrate plasma). After allowing the powder to stay in suspension in the plasma or serum for 15 minutes at room temperature, it was removed by high speed centrifugation (3600 g for 15 mins.). The absorbed plasma or serum was pipetted off, taking care not to resuspend the barium.

Aluminium Hydroxide Gel was made according to the method of Biggs & Macfarlane (1953).

For absorption of plasma or serum 0.1 ml. gel was added to 0.9 ml. plasma or serum, mixed thoroughly and allowed to stand for 3 minutes at 37°C. The aluminium hydroxide was removed by high speed centrifugation at 3400 g for 15 minutes. The supernatant plasma or serum was pipetted off.

Imidazole Buffer (Mertz & Owen 1940)

1.72 gm. Imidazole was dissolved in 90 ml. N/10 HCl and diluted with water to 100 ml.

Citrate Saline (0.85%)

0.85% saline containing 0.0075M trisodium citrate

Dextrose Acetate Saline (DAS) (Tullis 1953b)

This fluid, used for washing platelets free from plasma, was made up as follows:

L-Dextrose 5 gms.

Sodium acetate 0.2 gms. ($CH_3.COONa.3H_2O$).

Sodium chloride 0.8 gms.

Fresh distilled water to 100 ml.

This wolution was sterilised in small volumes by autoclaving for 10 minutes at 15 lbs. per square inch.

It was found convenient to sterilise and store this balanced salt solution in 20 ml. amounts and to discard any unused solution after each experiment as contamination frequently occurred when the bottle was opened.

Gelatine (Davis Sparkling Granulated Gelatin. Davis Gelatine Ltd., 29 Mitre Street, London, E.C.3.)

This was dissolved into warmed DAS to make 2 or 5% solutions w/v.

Bovine and Pig Antihaemophilic Globulin Concentrates

These were provided by Dr. E. Bidwell. They were prepared by the methods described by her in 1955. (Bidwell 1955a & b). In the present experiments the strength of any given solution is expressed as weight of protein/ml. This was not a true expression of the actual antihaemophilic globulin (A.H.G.) activity of any batch but provided a convenient method of experimental comparison of one species concentrate with another. The lyophilised concentrates were dissolved in an appropriate volume of distilled water. Any subsequent dilutions were made with isotonic saline.

Human Thrombin

This was prepared from human plasma using the method of Biggs & Macfarlane (1953).

The strength of thrombin in the final lyophilised extract was 23 thrombin units/mgm. For every mgm. of thrombin 1 ml. distilled water was added to reconstitute the the thrombin into solution (23 u/ml). Any further dilutions were made with isotonic saline.

Human Fibrinogen was prepared according to the method of Jaques (1943). (Quoted by Biggs & Macfarlane 1953, p.342).

Bovine Thrombin. Maw's Topical, 200 u/ml. This was dissolved in the provided diluent and diluted for use in 0.85% saline.

Glass Contact

Small glass ballotini, 0.1 mm. in diameter, were acid washed in dilute hydrochloric acid and subsequently washed x10 in distilled water.

In order to augment glass contact surface experimentally, an equal volume of glass ballotinae was added to 1 mL amounts of platelet suspension, plasma, or whole blood.

Siliconed Glass Beads were siliconed as ordinary glass tubes (see Appendix A, p. 2).

Brain Extracts

Saline Extract of Brain

The meninges were removed from a normal human brain and both cerebral hemispheres less cerebellum, pons and medulla were emulsified in 1500 ml. 0.85% saline in a 'Waring Blender'.

Acetone Extract of Brain was prepared by the method of Biggs and Macfarlane (1953).

Chloroform Extract of Brain was prepared by the method of Bell & Alton (1954).

Russell's Viper Venom. ('Stypven'. Burroughs Wellcome Ltd.).

The lyophilised extract was dissolved in the solvent provided and subsequent dilutions made with isotonic saline.

Zeo Carb. 215 (Permutit)

Cation ion exchange resin. 100 mgms. of this resin were added to each 1 ml. serum and allowed to act for 60 minutes at room temperature. Such sera was found to contain 0.9 - 1.8 mgm. calcium/100 ml. and to have a pH of 5.4 - 5.8 (Doran pH meter)

after adsorption for this period.

pH was determined by using the Doran Electric pH meter. Rough determinations were made using B.D.H. universal indicator. It was found convenient to add 1 drop of test solution to 1 drop indicator on a white tile which had been thoroughly washed in neutral distilled water beforehand.

Platelet Fixation

0.1 ml. of platelets were added to 1 ml. 15% neutralised salt formalin. After 5 minutes the platelets were spun down at 3600 g. and the supernatant fixative removed. The platelets were resuspended in 0.2 ml isotonic dextrose acetate-saline for microscopic examination.

Phase Contrast Microscopy

The morphological changes of platelet viscous metamorphosis and agglutination were observed in every instance by phase contrast microscopy. For routine morphological study, a Cooke, Troughton, & Sims phase contrast microscope was used using X200, X400 and X950 magnifications. The light source was conventional unfiltered direct illumination from a CTS lamp.

Phase Contrast Photomicrography

This was performed either using the above microscope or a Zeiss Phase Contrast Microscope (access to the latter was granted by Dr. Piris of the Nuffield Eye Laboratory). Photographs were taken using 35 mm. film in a reflex Praktiflex camera.

Kodak 'Rekordat' microfilm was used and developed in fine grain developer (Promicrol - May & Baker). Developing time 12 mins. at 68°C. This allowed 50% more developing time in order to achieve adequate contrast in the negative.

The films were fixed, washed and dried by conventional methods. Kodak 'Recordat' is ideal film for 35 mm. photomicrography as its very fine grain allows good enlargements to be produced.

Enlargements were made on Kodak single weight glossy bromide paper.

Developer. Johnstone Universal diluted as for bromide paper. Fixation using 'Amfix'.

(The prints, used as illustrations in this thesis, were printed by Mr. A.M. Blackwood of the Department of Anatomy, University of Oxford, from negatives provided by myself).

The Method Used to Study the Morphological Changes of Platelet Viscous Metamorphosis and Agglutination

As these changes evolved in the experimental systems used in their study, serial samples were removed and either examined immediately or fixed (Appendix A, p. 8) for subsequent study.

A drop of either preparation was added to thoroughly cleaned glass slides and covered with a clean coverslip. In order to obtain satisfactory phase contrast the field to be examined had to be thin and all movement inhibited. This was achieved by inverting the slide plus plasma and coverslip

onto filter paper and applying firm pressure for 10 seconds with a clean cloth on the under surface of the slide. Excess plasma was expressed onto the filter paper. The preparation was now placed, coverslip uppermost, on the microscope stage and examined. If prolonged examination was required the preparation was sealed with a vaseline-paraffin wax mixture. Phase contrast microscopy (Appendix A, p. 8) was used both for visual and photographic study of these changes.

Relative Centrifugal Force or g was determined according to the following formula:

$$g = \frac{(2\pi N)^2 R}{980 \times (60)^2}$$

N = revolutions per minute

R = radius of centrifuge head (spindle to centre of bucket) in cms.

Platelet Counts

These were performed by the method of Brecher & Cronkite (1950) using 1% ammonium oxalate as diluent.

A 1/20 dilution of capillary or venous blood, platelet rich plasma or washed platelet suspension was made using the above diluent.

An improved Neubauer counting chamber was filled with the diluted platelet suspension and the number of platelets in 160 squares each 1/400 of a square mm., counted using either direct microscopy or, in all experimental work, phase contrast microscopy. The depth of the counted area was 0.1 mm. Normal range 2-300,000/cu.mm.

Bleeding Times were performed by themethod of Ivy (Biggs & Macfarlane 1953). Normal - up to 5 minutes.

Occasional bleeding times on patients under the care of the Nuffield Medical Unit were performed by Duke's method (Duke 1912).

Capillary fragility was estimated by the application of a sphygmomanometer cuff around the upper arm at a pressure of 90 mm. of mercury for 5 minutes. After releasing the pressure the number of fresh petechiae appearing in 1 minute in an area 6 cms. in diameter over the antecubital fossa were counted. Abnormal capillary fragility was considered to exist if more than 10 fresh petechiae were present in this area

Whole Blood Clotting Time was performed by the method of Lee & White (1913). Normal - up to 10 minutes.

Prothrombin Consumption Index was derived from the results of the method of Merskey (1950). Normal 0 - 20%. (Originally 0 - 40%).

Thromboplastin Generation Test was performed as described by Biggs & Douglas (1953). Test plasma or serum fractions were compared with normal controls in each instance.

Platelet Rich Native Plasma

Venous blood was obtained using a 30 ml. siliconed or paraffin oiled syringe and clean No. 1 stainless steel needle. A rapid clean venepuncture was essential.

The blood was added immediately to cooled siliconed tubes and cooled rapidly by immersion of the tube into melting crushed ice contained in a thermos flask. No anti-coagulant was added at any stage. After 5 minutes cooling, the cooled blood was transferred to a refrigerated centrifuge previously cooled to 0°C. The red cells were separated by spinning at 1400 g for 10 minutes at 0°C and the platelet rich supernatant plasma transferred to a further cooled siliconed centrifuge tube in crushed ice; a siliconed pipette was used for transfer.

Platelet-Free Native Plasma

The whole blood was obtained as above and transferred to the refrigerated centrifuge. To free the plasma from all but the occasional platelet the blood was spun at 20,000 g for 15 minutes at 0°C in a siliconed high speed centrifuge tube. The platelet-free native plasma was transferred to a further siliconed tube in crushed ice.

It was also possible by this method to obtain native plasma free of chylomicra. These minute fat particles rise to the surface on high speed centrifugation. On cessation of centrifugation, the infranatant plasma is removed rapidly before the fat resuspends in the plasma.

Both platelet rich and platelet-free native plasma remained unclotted for several hours if kept at 0°C.

Native Plasma

Both platelet-rich and platelet-free native plasma would clot when added to glass tubes at 37°C. The clotting time of platelet free plasma was prolonged.

Platelet-Rich Citrate Plasma

Venous blood was obtained using a paraffin oiled or siliconed syringe and No. 1 stainless steel needle.

9 ml. venous blood was added to 1 ml. 3.8% sodium citrate in a siliconed centrifuge tube and mixed thoroughly. The decalcified blood was centrifuged at 1400 g for 15 minutes at room temperature and the platelet-rich plasma separated and placed in a siliconed tube.

Platelet-Rich Oxalate and Disodium Versene Plasma

These were prepared in a similar manner to platelet-rich citrated plasma.

(a) 9 ml. venous blood was added to 1 ml 1.34% sodium oxalate

(b) 9.7 ml. venous blood was added to 0.3 ml 4.5% disodium versene.

Siliconed glass was used throughout.

Washed Platelet Suspensions

Platelets were separated from platelet rich disodium versene plasma in a siliconed conical centrifuge tube by spinning at 420 g for 15 minutes. The platelets were packed into a button at the bottom of the tube and the supernatant plasma pipetted off. The final traces of plasma were removed by inverting the tube on filter paper for

5 minutes. A volume of washing fluid (dextrose acetate saline p. 5 unless otherwise stated) equal to half the original volume of plasma was added to the platelets, and the latter resuspended by agitation. Further centrifugation at 4200 g for 15 minutes repacked the platelets and the supernatant washing fluid was discarded.

This process was repeated until the desired number of washings had been performed.

The platelets were resuspended finally in a sufficient volume of saline or dextrose-acetate-saline to produce a final platelet density of 200-300,000 platelets /cu.mm.

Active Normal Serum (see Chapter IV). (Active VM factor)

Platelet-rich native plasma prepared as on p. was allowed to clot at 37°C and following the appearance of fibrin was incubated for 2 hours at 37°C to destroy any unused thrombin. The fibrin was removed and compressed mechanically in order that all serum was expressed from the clot. This serum was tested for its ability to induce VM in normal washed platelets.

Platelet Agglutination Test

A test suspension of washed platelets was prepared from normal blood. The platelets were washed x3 in dextrose-acetate-saline (Appendix A, p. 5) and the final resuspension made in dextrose-acetate-saline containing 2% gelatine (Appendix A, p. 6).

Test Serum was prepared from whole blood derived from the case

to be investigated. 10 - 20 ml. venous blood was collected into sterile universal containers and stored at 37°C for 18 hours. The serum was separated and, either tested immediately, or stored at -15°C before testing. On the day of testing the serum was heated to 56°C for 1 hour in a thermostatically controlled water bath. Then each sample was absorbed with barium sulphate powder to remove all residual prothrombin, together with Christmas factor and factor VII. The powder was added in the proportion of 200 mgms/ml serum and was allowed to act for 15 minutes at room temperature. The barium powder was removed by centrifuging at 3600 g for 15 minutes.

0.2 ml. treated test serum was added to 0.2 ml washed normal platelet suspension and incubated at 37°C for 2 hours. The contents of each tube was then examined for the presence or absence of platelet agglutination by phase contrast microscopy. It was found that any longer incubation would produce non-specific agglutination. Thus, if it was inconvenient to read the results at the end of the initial period of incubation, the tubes were placed in the refrigerator at 0-4°C. At this temperature the gelatine set solid and preserved the platelets in their agglutinated or non-agglutinated state. The tubes were reincubated at 37°C for 15 minutes prior to reading the results.

Sera for testing were stored until at least 3 samples were available and each serum was tested against four separate suspensions of normal platelets. In addition at least one normal control serum was tested against the same suspensions as in the following diagram.

Serum	Normal Platelet Suspension				From the pattern of positive agglutination tests the final results were recorded as follows
	1	2	3	4	
Test Serum 1	-	-	-	-	= Negative
2	+	-	-	-	= Negative
3	+	+	-	-	= Positive (iso-agglutinin)
4	+	+	+	+	= Positive (pan agglutinin)
Control serum 1	-	-	-	-	All must be negative. Any positive result in control columns invalidated all results in that column
2	-	-	-	-	

Tests Used to Determine Possible Sensitivity to Drugs in Cases of Thrombocytopenic Purpura

In cases of thrombocytopenia, where it was suspected that drug sensitivity played a part in the aetiology of platelet destruction, blood was tested in vitro using the following techniques.

Platelet Agglutination Test.

The patient's serum was tested as in the conventional agglutination test with the exception of the addition of a

solution of the suspected drug in 0.1 ml. amounts.

In patients whose platelet count had returned to normal following withdrawal of the drug, the following tests were also performed.

(1) Rapid Platelet Agglutination Test

To 0.5 ml. platelet rich versene plasma was added 0.1 ml. of a solution of the suspected drug. The mixture was incubated at 37°C for 60 minutes. The presence or absence of platelet agglutination was then determined. Control samples of normal platelet-rich versene plasma plus drug or saline and patient's platelet-rich versene plasma and saline were also tested. If drug sensitivity existed strong agglutination developed within 15 minutes.

(2) Clot Retraction Test

1 ml. amounts of the patient's rich plasma were added to 1 ml. M/40 calcium chloride together with 0.1 ml. of a solution of the suspected drug. The plasma was allowed to clot at 37°C and incubated for 1 hour at that temperature. The degree of clot retraction was then recorded as normal or inhibited. It was apparent that if clot retraction were inhibited due to drug sensitivity, it was inhibited completely. Control normal platelet-rich plasma was tested in parallel. In all these experiments test drugs were made up in solution to give a final concentration of 10 mgms/ml. If partially insoluble, each drug was made up as a saturated solution and used for testing. All drugs were dissolved in dextrose-acetate-saline.

pH

Certain drugs, when dissolved in solution, were acid in reaction. These acid solutions were capable of agglutinating both washed platelet suspensions and platelets in versene plasma non-specifically. Further, they inhibited clot retraction in recalcified platelet-rich plasma. When these acid solutions were encountered, the drug was redissolved in dextrose acetate saline containing an equal volume of buffered Imidazole buffer (pH 7.3. Appendix A, p. 5). The mild non-specific platelet agglutination resulting from the addition of this buffer (p. 131) could be ignored in these tests where a positive result was accompanied by gross platelet agglutination.

Tanned Red Cell-Platelet Protein Agglutination Test

(Kissmeyer Nielsen 1953)

Red Cells.

Human group O erythrocytes free from platelets were obtained from defibrinated normal blood. These cells were washed three times in 0.85% saline and resuspended in 0.85% saline (buffered to pH 7.2 with phosphate buffer) to give a 2.5% concentration of cells.

Tannic Acid Treatment of Red Blood Cells

One volume of this red cell suspension was added to one volume of tannic acid (B.D.H. 1/40,000 w/v in fresh 0.85% saline). The mixture was incubated at 37°C for 10 minutes and the red cells separated from the tannic acid solution by

centrifugation and washed once in buffered saline (pH 7.2). The red cells were resuspended finally in 0.85% saline in a 2% concentration.

Antigenic Extract (Platelet Protein)

Platelets were separated from platelet rich citrate plasma and resuspended in 0.85% saline (glass tubes were used instead of siliconed tubes). The final resuspension of platelets was made in 0.85% and frozen at -20°C and thawed. The process was repeated three times and the platelet debris removed by spinning at 4200 g for 15 minutes. The supernatant was used as the 'antigenic extract'.

Combination of Tanned Red Cells with Platelet Protein

One volume of a 2.0% suspension of tanned red cells was added to one volume of protein extract and one volume of phosphate buffer to give a pH of 6.4.

The mixture was allowed to stand for 30 minutes at room temperature. Then the red cells were separated by centrifugation and washed twice in normal rabbit serum diluted 1/100 in 0.85% saline. The final resuspension of these red cells was made in 1/400 normal rabbit serum to give a 1% concentration.

Test Serum was inactivated at 56°C for 30 minutes.

Test

Doubling dilutions of 0.1 ml volumes of test sera were made in saline and 0.1 ml. 1% tanned-platelet protein red cells was added to each dilution.

The mixtures were incubated at room temperature for 2 hours and then stored at 0 - 4°C for 18 hours or overnight. The presence of absence of red cell agglutination was then determined microscopically.

Controls

(1) Tanned-platelet-protein red cells + normal serum.

(2) Normal untreated red cells + test serum.

(3) Tanned normal red cells + test serum.

Platelet Agglutination Test 'Methode d'agitation'

(Dausset 1954)

Platelet-rich versene normal plasma was prepared as described in Appendix A, p. 13.

Serum from the patient or control was heated at 56°C for 30 minutes.

Test

1 ml. test serum was added to 0.1 ml platelet-rich normal versene plasma contained in a siliconed glass tube. The mixture was agitated in a Kahn shaker for 30 minutes at 37°C. Aliquot samples were then removed and observed by phase contrast microscopy.

The presence or absence of alterations in platelet morphology were recorded.

In Dausset's original paper he stated that he added 10 ml test serum to 0.5 ml platelet rich plasma. These volumes were found to be excessive and the above concentration of platelets produced an optimum density for microscopic examination.

Complement Fixation Test for Platelet Antibodies

Antigen. A three times washed suspension of normal human platelets was prepared from platelet-rich versene plasma as described on p. 13.On final resuspension the platelets were resuspended in the least diluent to give a concentration of 500,000/cu.mm.

Test Serum from patients, normals, or rabbits was heated at 56°C for 30 minutes to inactivate natural 'Complement'.

Complement was provided as fresh guinea pig serum used undiluted.

Haemolytic System

Sheep red blood cells separated from oxalated blood were washed x3 in saline and following the final wash the red cells were packed by centrifuging at 800 g for 20 minutes.

0.75 ml. packed red cells were added to 25 ml. saline to give a 3% suspension.

0.13 ml. haemolytic serum was diluted in 25 ml. saline.

Equal volumes of the 3% sheep cell suspension and haemolytic serum were mixed and incubated at 37°C for 20 minutes and used on the day of preparation only.

To detect the presence or absence of Complement, 2 volumes of this suspension were added to 1 volume of each test mixture.

(Wellcome Haemolytic serum for sheeps cells: Burroughs Wellcome & Co. Ltd.)

Test

0.5 ml. test serum was added to 0.1 ml. platelet suspension together with 0.1 ml. Complement. The mixture was incubated for 1 hour at 37°C and 1 hour at room temperature. Each tube was then spun at high speed to remove all platelets and 0.2 ml. of the supernatant was diluted $\frac{1}{2}$ and $\frac{1}{5}$ and, in doubling dilutions of these, to a titre of 1/512.

To each dilution was added 0.4 ml. of the haemolytic system and after incubation at 37°C for 30 minutes the titre of residual Complement was determined as described in Appendix B, p. 4.

The following control mixtures were tested in parallel:

Control (1). Test Serum 0.5 ml.
Saline 0.2 ml.

" (2) Complement 0.1 ml.
Saline 0.6 ml.

" (3) Saline 0.5 ml.
Platelet Suspension 0.1 ml.
Complement 0.1 ml.

" (4) Test Serum 0.5 ml.
Complement 0.1 ml.
Saline 0.1 ml.

" (5) Serum 0.5 ml.
Complement 0.1 ml.
Washed human red cells 0.1 ml.

Mixtures 3 and 4 were designed to measure any anti-Complementary activity on the part of the platelets or serum.

Mixtures 2 and 5 gave a control for a comparison of the amount of Complement consumed in the actual test mixture.

In all examples where human sera tested in this system no Complement was found to be fixed by the serum-platelet mixtures.

When rabbit antisera containing a high titre of human platelet agglutinin were tested, Complement was fixed, there being no residual Complement left following incubation of the test mixture. Control mixtures contained residual Complement.

The results of such an experiment is illustrated below.

Mixture	Titre of Residual Complement
Test (Rabbit Serum)	0
Control 1	0
Control 2	1/32
Control 3	1/32
Control 4	1/16
Control 5	1/16

Direct Coombs' Test

Suspensions of washed normal human platelets were prepared as on p.A.13. To 0.1 ml. of these suspensions were added 0.1 ml. amounts of anti-human globulin sera diluted 1/2, 1/10, and 1/100. The mixtures were incubated for 30 minutes at 37°C and the platelet suspension examined by phase contrast microscopy.

Mixed Red Cell Platelet Agglutination Test

(Coombs, Marks and Bedford 1956)

Method

Platelet Suspension

A washed suspension of normal human platelets was prepared as described in Appendix A p.3 from Group O donors.

Test Sera from normals and patients with apparent platelet agglutinins were treated by heating at 56°C for 30 minutes only.

Sensitised Red Blood Cells

Red blood cells were separated from venous blood samples from Group O Rhesus positive donors. These cells were washed three times in dextrose acetate saline solution (DAS) and finally resuspended in a 2% solution in DAS.

To 1 ml. of these cells was added 1 ml. of a 1/10 dilution of anti Rhesus serum (anti D) containing an incomplete non-agglutinating antibody, (supplied by Dr. M.M. Pickles). The mixture was incubated for 1 hour at 37°C. The sensitised red cells were then separated by centrifuging and washed x3 in DAS.

Anti-human Globulin Rabbit Serum (diluted 1/10) was supplied by Dr. M.M. Pickles.

Test

3 drops of platelet suspension were added to 3 drops of each test sera and the mixture incubated for 1 hour at 37°C.

The platelets were then deposited by centrifuging at 2800 g for 5 minutes. The supernatant fluid was removed and the platelets in each tube washed three times in DAS and finally resuspended in 1 drop DAS. To each tube was added one drop sensitised red blood cells and one drop diluted anti-human globulin sera.

The mixtures were centrifuged at 2800 g for 2 minutes and the platelet-red cell mixture resuspended.

Each tube was examined by phase contrast microscopy for the presence of mixed red cell platelet agglutination.

APPENDIX B

EXPERIMENTS

Experiment 1

Purpose. To study the relationship of platelet viscous metamorphosis to fibrin formation in normal and abnormal plasma.

Method

Platelet-rich native plasma was prepared as described in Appendix A, p.11, from normal blood and from blood derived from patients with known specific congenital or acquired coagulation defects.

In every instance 30 ml. syringes and No. 1 stainless steel needles were used in order to standardise experimental conditions.

0.5 ml. amounts of platelet-rich plasma were allowed to clot at 37°C in glass tubes of uniform diameter, while being agitated continuously. This agitation promoted platelet VM and the onset of this phenomenon was recorded in relation to the time the platelet-rich native plasma was added to glass tubes at 37°C and to the appearance of fibrin. The results are recorded in Table I.

Experiment 2

Purpose. To study the effect of various anticoagulants on platelet VM in normal platelet-rich native plasma.

Method

The following anticoagulants were added in 0.5 ml. amounts to 0.5 ml. amounts of platelet-rich/native plasma to
normal

give final concentrations as defined in Table II. The influence of these agents in VM was recorded (Table II).

Sodium citrate
Sodium oxalate
Disodium versene
Heparin
Thrombodym
Soya bean trypsin inhibitor

} see Appendix A, pp. 2 and 3

Experiment 3

Purpose. To decide the role of calcium in VM.

Method

Platelet-rich citrate, oxalate and disodium versene plasma were prepared as described in Appendix A, p.13.

0.5 ml. of each type of platelet-rich plasma was added to 0.5 ml. M/40 calcium chloride. The tubes were agitated at 37°C and the time of onset of VM and fibrin formation recorded in relation to the addition of calcium. The results are recorded in Table III.

Experiment 4

Purpose. To determine the optimal Ca^{++} strength for platelet VM.

Method

0.5 ml. platelet-rich citrated plasma was added to 0.5 ml. samples of M/40 $CaCl_2$ solutions diluted in doubling dilutions to give final concentrations as defined in Table IV. Each mixture was agitated at 37°C and the time of onset of VM recorded. The presence or absence of fibrin was observed after 30 minutes' incubation. The results are recorded in Table IV.

Experiment 5

Purpose. To compare the sequence of VM in normal platelet-rich native plasma and in normal platelet-rich recalcified plasma.

Method

Venous blood was obtained from 5 normal volunteers and both platelet-rich native plasma and platelet-rich citrate plasma prepared according to the methods detailed in Appendix A, p. 13.

To 0.5 ml. platelet-rich native plasma was added 0.5 ml. 0.85% saline and to 0.5 ml. platelet-rich citrate plasma was added 0.5 ml. M/40 $CaCl_2$. Both specimens were allowed to clot at 37°C in glass tubes of identical size and the onset of VM and fibrin formation observed, and the times from the onset of clotting ~~in both samples~~ were recorded (Table V).

Experiment 6

Purpose. To determine the relationship of Complement to platelet VM.

Method

Both platelet-rich and platelet-free samples of native plasma were obtained from both normal and abnormal plasma (Appendix A, pp. 11 & 12 and Table VI).

1 ml. amounts of both platelet-rich and platelet-free native plasmas were allowed to clot at 37°C in glass tubes of identical size. After the formation of fibrin the clotted plasma was incubated for 1 hour at 37°C. The fibrin was then removed and the resultant serum titrated for its content of residual Complement.

This titre was estimated by making doubling dilutions of sera diluted $\frac{1}{2}$ and $\frac{1}{3}$ in normal saline. To each dilution was added 2 volumes of 1.5% suspension of sensitised sheep cells. (Appendix A, p.21). After 30 minutes' incubation at 37°C the tubes were spun at 1400 g for 5 minutes and the percentage haemolysis determined in each tube. 0.5 ml. of each supernatant was diluted to 4.5 ml. with 0.04% ammonia. The degree of haemolysis in each tube was determined using the M.R.C. grey wedge photometer. 100% haemolysis standard was prepared by adding 2 volumes of sensitised sheep cells to 1 volume distilled water. 0% standard contained distilled water only.

Experiment 7

Purpose. To determine the effect of platelet numbers on the activity of serum VM factor.

Method

Platelet-rich and platelet-free samples of normal native plasma were prepared as described in Appendix A, pp. 11 & 12, and 1 ml. samples were allowed to clot in glass tubes of identical size at 37°C. Following the formation of fibrin the clotted plasma was incubated for 1 hour at 37°C to destroy thrombin. Its ability to induce VM in twice-washed normal platelets was assayed by titrating the activity in doubling dilutions of sera. 0.2 ml. serum was added to 0.2 ml. of a twice-washed platelet suspension prepared as in Appendix A, p.13. Tubes of identical size were used for all experiments. The mixture was agitated at 37°C and a positive result was

recorded if typical VM had developed within 60 seconds of the test sera being added to the platelets.

The titres of VM activity in the sera derived from platelet -rich and platelet-free plasma are recorded in Table VII.

Experiment 8

Purpose. To establish the effect of washing on the ability of platelets to react with serum VM factor.

Method

Platelet-rich versene plasma was prepared from 8 separate samples of normal blood as in Appendix A, p.13.

The platelets in each sample were separated from the plasma and washed repeatedly in dextrose acetate saline (Appendix A, p.5). After each washing 0.1 ml. platelet suspension was added to a tube containing 0.1 ml. active normal serum prepared as in Appendix A, p.14.

The tubes were agitated at 37°C and the onset of typical VM in 60 seconds recorded (Table VIII). To ensure that the platelet suspensions were not successively diluted with each wash the volume of resuspending DAS was reduced by 0.1 ml. after each wash.

Experiment 9

Purpose. To establish the minimum platelet density for normal VM.

Method

A twice-washed normal platelet suspension was prepared from platelet-rich versene plasma (Appendix A, p.13). A

series of doubling dilutions of this platelet suspension was made in DAS and the total number of platelets/cu.mm. in each suspension determined by the method of Brecher & Cronkite (1950) (Appendix A, p.10). To 0.2 ml. of each dilution of this platelet suspension was added 0.2 ml. active normal serum and the mixture agitated at 37°C. The presence or absence of typical VM after 10 minutes was recorded (Table IX) following both naked eye observation and phase contrast microscopy.

Experiment 10

Purpose. To assess the VM activity of certain abnormal sera and to observe the ability of platelets derived from blood with known coagulation defects, to undergo typical VM when exposed to normal active sera.

Method

(i) Sera were prepared from platelet-rich samples of normal and abnormal plasma (Appendix A, p.14) derived from blood with known congenital or acquired deficiencies of specific coagulation factors (see Table X). 0.2 ml. amounts of these sera were added to 0.2 ml. amounts of a twice-washed normal platelet suspension (Appendix A, p. 13) and the presence or absence of typical VM was observed after the mixture had been agitated at 37°C for 60 seconds.

The results are recorded in Table X.

(ii) Twice-washed platelet suspensions were prepared from platelet-rich versene plasma derived from patients with specific congenital or acquired coagulation defects (see Table X). The

method used was identical to that described for the preparation of normal washed platelet suspensions (Appendix A, p.13). To 0.2 ml. of these varying suspensions was added 0.2 ml. normal active serum and the presence or absence of typical VM was observed after the tube had been agitated at 37°C for 60 seconds.

Results are recorded in Table X.

Experiment 11

Purpose. To decide the relationship of serum VM factor to thrombin.

Method

Thrombin may be defined as that substance which converts fibrinogen to fibrin.

Therefore to discover whether serum VM factor was related to existing or generated thrombin in serum-platelet mixtures, 0.2 ml. additions were made to 0.2 ml. amounts of normal active serum and human fibrinogen as defined in Table XI. These mixtures were incubated for varying periods and the presence or absence of fibrin recorded.

The results of these experiments are recorded in Table XI.

Human fibrinogen
Human Thrombin
} see Appendix A, p.6.

Experiment 12

Purpose. To determine the relationship of Complement to serum induced VM.

Method

Samples of 5 fresh active normal sera were tested for their Complement activity before and after exposure to normal

twice-washed platelet suspension (Appendix A, p.13). 0.5 ml. of each serum was added to 0.5 ml. DAS containing a 2% suspension of washed human red cells, and was incubated at 37°C for 10 minutes;(B). 0.5 ml. of each serum was added to 0.5 ml. platelet suspension and after agitation at 37°C for 10 minutes typical VM evolved. The platelet masses were then removed by centrifuging at 1400 g. for 15 minutes (A).

The titre of residual Complement in each sample of serum was determined both in the control and test sera as in Experiment 6 (Appendix B, p.3). The results are recorded in Table XII.

Experiment 13

Purpose. To determine the properties of the viscous metamorphosing factor in serum.

Method

0.2 ml. active normal serum was added to 0.2 ml. of a twice-washed normal platelet suspension, before and after varying treatments, or together with 0.1 ml. of additions.

Serum Treatment

Heat treatment. 0.2 ml. amounts of serum were added to tubes and placed in a 56°C water bath for 30 or 60 minutes prior to testing.

Absorption with Aluminium Hydroxide gel ($Al(OH)_3$ and Barium Sulphate Powder ($BaSO_4$))

1 ml. amounts of sera were absorbed with these inorganic salts as detailed in Appendix A, pp. 4 and 5.

Zeo carb. (Appendix A, p.7).

100 mgms. cation ion exchange resin was added to 1 ml. active normal serum and allowed to act for 10 minutes at 37°C.

Platelet-Absorbed Serum

0.5 ml. active normal serum was added to 0.2 ml. packed twice-washed platelets (Appendix A, p.13). The latter were resuspended in the serum and after agitation at 37°C for 15 minutes the platelet masses were spun off at 1400 g for 15 minutes. 0.2 ml. of the supernatant serum was added to 0.2 ml. twice-washed platelet suspension and any residual VM activity determined.

Red Cell Absorbed Serum

Active normal serum was absorbed with twice-washed human red cells substituted for platelets.

Dialysed Serum

5 ml. fresh active normal serum was placed in a bag of dialysing membrane and dialysed overnight (18 hours) against 3 litres 0.85% saline at 0°C.

Following removal from the dialysis bag the serum was tested for VM activity.

Storage Active normal sera were stored in glass universal containers (i) at room temperature for 24 hours, (ii) at -12°C for 24 hours and (iii) at -12°C for 7 days. The residual VM activity was then determined.

Additions

Sodium citrate 1.9%
Sodium oxalate 0.67%
Disodium versene 0.8%
Heparin 5 u/ml
Thrombodyn 2.5%
Soya bean trypsin inhibitor 5 mgm/ml

have been described in Appendix A, p.243 .

The results of these experiments are recorded in Table XIII.

Experiment 14

Purpose. To concentrate serum VM factor.

Method

Active fresh normal serum was prepared as detailed in Appendix A, p.14. Its ability to induce typical VM in washed normal platelets was confirmed. From this serum various protein extracts were prepared and their ability to induce VM in washed platelets observed.

Globulin Fraction

2 ml. of active serum was added to 18 ml. of distilled water which had been acidified by carbon dioxide gas.

After the mixture had stood 15 minutes at room temperature the precipitate obtained was separated by centrifugation and washed once in acidified distilled water.

The precipitate was dissolved in 1 ml. 0.85% saline and the pH adjusted to 7.0 by 1% ammonium carbonate.

0.2 ml. of this extract was added to 0.2 ml. of a suspension of washed platelets and following agitation at 37°C, the presence or absence of typical VM observed. The results are recorded in Table XIV.

Ammonium Sulphate Fractions

10 ml. active serum was added to 10 ml. 50% saturated ammonium sulphate. The precipitate obtained was separated by centrifugation (0-25% ammonium sulphate fraction), and the clear supernatant removed.

To 10 ml. of this supernatant was added a further 10 ml. 50% saturated ammonium sulphate. Again the precipitate obtained was separated (25 - 33% ammonium sulphate fraction) and the supernatant removed.

To 10 ml. of the second supernatant was added 1 gm. ammonium sulphate. The third and last precipitate was separated (33 - 50% ammonium sulphate fraction) and the supernatant discarded.

All three precipitates were dissolved separately in 5 ml. amounts of 0.85% saline and each solution dialysed in dialysis bags against 0.85% citrate saline at 0 - 4°C.

The volumes of the resultant dialysed fractions were adjusted to 10 ml. with 0.85% saline. 0.2 ml. samples of each dialysate were tested for their ability to induce VM in suspensions of washed human platelets.

The results are recorded in Table XIV.

Phosphate Fractionation

Volumes of 3M phosphate solution were added to 5 ml. samples of active normal serum to give final concentrations of 1.0M, 1.5M and 2.0M of phosphate.

The 3M phosphate was made up as follows:

$K.H_2PO_4$ 817.0 gms.
KOH 168.0 gms.
Distilled water to 2 litres.

The final concentrations were made by the following dilutions:

Serum/ml	Distilled water/ml	3M Phosphate /ml	Final Phosphate Conc.
5	5	5	1.0
5	2.5	7.5	1.5
5	0	10	2.0

The precipitates obtained with each concentration of phosphate were separated by centrifugation at 3400 g. for 30 minutes. The infra- or supernatant was removed and discarded.

Each precipitate was dissolved in 0.85% saline and dialysed overnight against 0.85% saline at 4°C.

The ability of each dialysed solution to induce VM in suspensions of washed normal platelets was assessed.

The results are recorded in Table XIV.

Experiment 15

Purpose. To confirm the composition of Bergsagel's VM factor (Bergsagel 1956).

Method

An incubation mixture was made of pig antihaemophilic globulin (1 mgm protein/ml) 0.1 ml. together with 0.1 ml. M/40 calcium chloride and 0.1 ml. addition.

The additions were composed of 1/5 dilutions of varying aged sera derived from patients with known specific coagulation defects and normal heat treated and barium sulphate absorbed serum.

After incubation of these mixtures for 20 minutes at 37°C, 0.2 ml. samples of the varying test incubation mixtures were tested for their ability to induce typical VM in 0.2 ml. of a twice-washed human platelet suspension (Appendix A, p.13).

The results are recorded in Table XV.

Experiment 16

Purpose. To define the properties of Bergsagel's VM factor.

Method

An incubation mixture was prepared, consisting of 1 ml. amounts pig antihaemophilic globulin, aged normal serum and diluted 1/5 in saline M/40.

After incubation for 20 minutes at 37°C, 0.2 ml. of the incubation mixture was added to 0.2 ml. twice-washed normal platelet suspension before and after certain treatment or the addition of certain anticoagulants (0.1 ml.).

The incubation mixture was heat treated, absorbed with aluminium hydroxide gel, barium sulphate powder, platelets and red cells, and stored as in Experiment 13.

Additions of various anticoagulants were made in strengths as defined in Experiment 13.

The results are recorded in Table XVI.

Experiment 17

Purpose. To observe the effect of thrombin on washed normal human platelets.

Method

Human thrombin was prepared as detailed by Biggs & Macfarlane (1953).

Bovine thrombin. Maws Topical Thrombin 200 u/ml. was used.

0.2 ml. volumes of both human and bovine thrombins, in a concentration of 20 u/ml, were added to 0.2 ml. twice-washed suspensions of normal human platelets (Appendix A, p.13) together with 0.2 ml. of addition. Glass tubes of identical size were used and the mixtures agitated at 37°C for 15 minutes.

Any alteration in the morphology of the platelet suspensions was observed by phase contrast microscopy.

The results are recorded in Table XVII.

Experiment 18

Purpose. To observe the effect of certain anticoagulants on thrombin induced VM.

Method

0.4 ml. human thrombin, diluted to a strength of 1 u/ml., was added to 0.5 ml. platelet rich citrate, oxalate, or versene plasma together with 0.1 ml. 0.85% saline. Alternatively, an identical amount of thrombin was added to 0.5 ml. platelet-rich citrate plasma together with 0.1 ml. of varying anticoagulants (heparin 10 u/ml.; thrombodyn 2.5%; soya bean trypsin inhibitor, 10 mgm/ml.). In each instance the presence or absence of typical VM occurring at least 1 minute before fibrin formation was recorded.

The results are shown in Table XVIII.

Experiment 19

Purpose. To decide the ability of human thrombin to promote VM in abnormal platelet-rich plasma.

Method

Platelet-rich citrate plasma (Appendix A, p13) was prepared from patients with known specific congenital or acquired coagulation defects. To 0.5 ml. of each sample was added 0.4 ml. of human thrombin and the presence or absence of typical VM, developing 60 seconds prior to fibrin formation, recorded.

The results are detailed in Table XIX.

Experiment 20

Purpose. To determine the nature of thrombin VM co-factor.

Method

As it has been shown that human thrombin has no action on washed human platelets, it was possible to test any material for co-factor activity by addition to a mixture of human

thrombin and washed human platelets (Chapter V).

0.1 ml. human thrombin (7 u/ml.) was added to 0.4 ml. washed platelets together with 0.1 ml. addition.

The additions were

(i) saline 0.85%;

(ii) high spun platelet-free citrated normal plasma;

(iii) normal platelet-free oxalate plasma absorbed with barium sulphate;

(iv) aged serum (24 hours storage at 37°C, or 7 days at room temperature;

(v) pure human fibrinogen (Appendix A, p.6).

The presence or absence of typical VM, evolving at least 60 seconds prior to fibrin formation, was observed.

The results are recorded in Table XX.

Experiment 21

Purpose. To test for thrombin co-factor activity in platelet extracts.

Method

Human platelets were separated from normal platelet-rich citrate plasma by high speed centrifugation. To one sample was added 2 ml. distilled water and the suspension frozen solid in an ice salt mixture. This was then thawed rapidly at 37°C. The freeze-thawing was repeated x3 and the platelet debris removed by spinning at 20,000 g for 15 minutes. The supernatant was tested for co-factor activity.

Platelets separated from a second sample of platelet-rich citrate plasma were washed x3 in normal saline before being resuspended in distilled water

and exposed to the process of extraction. The platelet debris was again separated and the supernatant tested for co-factor activity.

0.1 ml. of both extracts was added to 0.4 ml. of a suspension of twice-washed normal human platelets together with 0.1 ml. of thrombin (7 u/ml.). The presence or absence of typical platelet VM evolving within 60 seconds of the addition of thrombin was observed.

The results are recorded in Table XXI.

Experiment 22

Purpose. To decide the relationship of thrombin VM co-factor activity to platelets.

Method

High spun platelet-free citrated plasma was prepared from (1) a fresh specimen of blood collected into acid citrate dextrose (disodium citrate 2 gm., dextrose 3 gm., water to 120 ml.).

(2) Stored blood collected into A.C.D. 21 days prior to testing and stored at 4°C.

0.1 ml. samples of these plasma were added to 0.4 ml. of a suspension of x2 washed normal human platelets together with 0.1 ml. human thrombin (7 u/ml.).

The presence or absence of typical VM evolving within 60 seconds of the addition of thrombin was observed.

The results are recorded in Table XXII.

Experiment 23

Purpose. To observe the effect of foreign surfaces on VM in platelet-rich normal and abnormal native plasma.

Method

Platelet-rich native plasma was prepared as in Experiment 1 from normal and abnormal plasma. 0.5 ml. samples of each plasma were allowed to clot either in glass or siliconed tubes of identical size at 37°C, and the time of onset of VM and the appearance of fibrin compared. The tubes were agitated as the plasma was allowed to clot.

The results are recorded in Table XXIV.

Experiment 24

Purpose. To observe the effect of foreign surface on the activity of VM factor in serum.

Method

1 ml. platelet-free samples of normal native and recalcified citrate plasma were allowed to clot at 37°C after contact with varying areas of glass and siliconed glass. The tubes were of identical size but the area of glass was increased by the addition of 1 ml. amounts of glass ballotini to one sample of each plasma.

Following fibrin formation the tubes were incubated for 2 hours at 37°C to destroy thrombin. The fibrin was removed and the VM activity of the resultant sera assayed by titration.

Doubling dilutions of each sera were made and 0.2 ml. of each dilution was added to 0.2 ml. of a suspension of normal human platelets.

The highest dilution of serum, which could produce typical VM of the washed platelets within 60 seconds, was recorded as the strength of the VM factor.

The results are detailed in Table XXV.

Experiment 25

Purpose. To define the effect of glass surface contact on normal human platelet suspensions.

Method

Suspensions of normal human platelets were prepared as follows:

(1) Platelet-rich citrate plasma
(2) " " versene plasma
(3) x2 washed suspension of platelets

} Appendix A

1 ml. samples of each suspension were agitated both in glass and siliconed tubes by rotation once every 3.5 seconds for 2 hours at room temperature.

The number of platelets /cu.mm. in each suspension was counted before and after agitation using the direct technique of Brecher & Cronkite (Appendix A, p.10), and phase contrast microscopy.

The results are recorded in Table XXVI.

Experiment 26

Purpose. To observe the effect of foreign surfaces on the morphology of normal human platelet suspensions.

Method

1 ml. samples of human platelet-rich citrate plasma and a x2 washed suspension of normal human platelets were added to

glass and siliconed tubes containing glass and siliconed beads respectively. All tubes were agitated for 1 hour at room temperature by rotation once every 3.5 seconds.

The morphological changes observed by phase contrast microscopy are recorded in Table XXVII.

Experiment 27

Purpose. To observe the effect of tissue thromboplastins on platelet VM.

Method

0.2 ml. amounts of tissue thromboplastin in the form of brain extracts prepared as detailed in Appendix A, p.7., were added to 0.4 ml. normal platelet-rich citrated plasma, or 0.4 ml. of a twice-washed normal platelet suspension. To each tube was added 0.2 ml. of addition in the form of saline or M/40 calcium chloride.

The morphology of any platelet changes were observed both microscopically and macroscopically. The results are recorded in Table XXVIII.

Experiment 28

Purpose. To observe the effect of adding Russell's Viper Venom to normal platelet suspensions.

Method

0.2 ml. Russell's Viper Venom ('Stypven' Burroughs Wellcome Ltd), was added in varying dilutions to 0.4 ml. normal platelet-rich citrate plasma or 0.4 ml. of a twice-washed suspension of normal human platelets. 'Stypven' represents viper venom in a 1/10,000 dilution; therefore when used

undiluted this material is tabulated at this dilution (Table XXIX).

Platelet changes were observed by phase contrast microscopy. The results are recorded in Table XXIX.

Experiment 29

Purpose. To determine the effect of animal antihaemophilic globulin concentrates on human platelets.

Method

Pig and bovine antihaemophilic globulin concentrates were prepared in varying strengths as defined in Appendix A, p.6., and in Table XXX.

0.2 ml. amounts of these concentrates were added to both a normal platelet-rich citrate plasma and a twice-washed suspension of normal platelets. The mixtures were incubated at 37°C for 15 minutes. Any alterations in platelet morphology were observed by phase contrast microscopy.

The results are recorded in Table XXX.

Experiment 30

Purpose. To observe the effects of animal antihaemophilic globulin on other heterologous platelet suspensions.

Method

Platelet-rich citrate plasma was obtained from venous blood derived from a pig, cow, rat, guinea pig, rabbit, mouse, cat, and dog.

0.2 ml. volumes of each of these plasmas were added to 0.2 ml. of either pig or bovine A.H.G. (see Experiment 29), (10 mgms/ml.). The mixtures were incubated at 37°C for

15 minutes and any alterations in platelet morphology observed by phase contrast microscopy.

The results are recorded in Table XXXI.

Experiment 31

Purpose. To study the effect of varying anticoagulants on the stability of platelet suspensions.

Method

Samples of venous blood were added to the following anticoagulants in siliconed tubes:

Tri-sodium citrate (3.8%) 1 part to 9 parts blood.

Sodium oxalate (1.34%) 1 part to 9 parts blood.

Disodium versene (4.5%) 1 part to 33 parts blood.

Heparin (10 u/ml. blood).

Platelet rich plasma was prepared from each sample and incubated in siliconed tubes at 37°C for 18 hours. Any alteration of platelets morphology was observed and recorded in Table XXXIII.

Experiment 32

Purpose. To decide the optimum medium for washing platelets free from plasma.

Method

Platelets were separated from 5 mls. platelet-rich versene plasma and washed three times as described in Appendix A, p.13, with 2.5 ml. amounts of the following solutions:

0.85% saline

0.85% citrate saline

5% glucose in 0.85% saline

5% glucose

Dextrose acetate saline. Appendix A, p.5.

Dextrose acetate saline (buffered to pH 7.3 with an equal vol. glyoxaline buffer).

1% disodium versene in saline.

After these washings each sample of platelets was re-suspended in 1 ml. volumes of their respective washing solutions. Any alteration in platelet morphology was observed and recorded in Table XXXIV.

Experiment 33

Purpose. To determine a suitable medium for preserving platelets in suspension.

Method

Platelets were separated from 2 ml. platelet-rich versene plasma and washed three times in dextrose acetate saline (Appendix A, p.13). Finally they were resuspended in 0.6 ml. volumes of the following solutions and 0.2 ml. volumes of each suspension incubated in siliconed tubes at 37°C, room temperature and 4°C respectively for 3 hours.

Saline 0.85%

Citrate saline 0.85%

1% Disodium versene in 0.85% saline

Glucose 5%

5% Glucose in 0.85% saline

Dextrose acetate Saline (Appendix A, p. 5)

2% Gelatin in DAS

5%Gelatin in 0.85% saline

20% bovine albumen

Human albumen 5% (Lister Institute)

Egg albumen 10% in 0.85% saline (v/v)

Human fibrinogen (100 mgms/ml 0.85% saline)

Any platelet agglutination that had resulted from this process was recorded.

The results are presented in Table XXXV.

Experiment 34

Purpose. To observe the effect of washing on the platelet-red-cell agglutination phenomenon.

Method

Normal venous blood from Group O Rhesus positive donors was collected into disodium versene and the platelet rich plasma separated (Appendix A, p.13)

0.2 ml. samples of a 10% suspension of red blood cells in plasma and in saline, after one, two and three washes, was added to 4 separate 0.2 ml. samples of platelet-rich plasma. All 4 samples were spun at 3600 g for 5 minutes and resuspended by tapping. The presence or absence of platelet or mixed platelet-red cell agglutination was observed in each tube.

This experiment was repeated adding red cells to platelet suspensions after they had been washed one, two or three times. Again, each mixture was centrifuged , resuspended and the presence of platelet or mixed platelet-red cell

agglutination observed.

The results are recorded in Table XXXVI.

Experiment 35

Purpose. To study the effect of trypsin and incomplete antibodies on platelet red cell agglutination.

Method

Normal red blood cells were separated from Group O Rhesus positive donors and washed three times in normal saline. The cells weremodified either by the use of trypsin or by sensitising with antiserum containing an incomplete anti-Rhesus (anti-D) antibody.

Trypsinised Red Blood Cells

A solution of trypsin (30 mgms/ml) was diluted 1/10 and 1 ml. added to 1 ml. packed washed red blood cells. The mixture was incubated for 30 minutes at 37°C. The red cells were then separated by centrifugation, washed three times in dextrose-acetate-saline (DAS) solution and finally resuspended in DAS to give a 10% suspension.

Sensitised Red Blood Cells were prepared as for the mixed red cell platelet agglutination test (Appendix A, p.24). The sensitised cells were resuspended to give a final concentration of 10% red blood cells in DAS.

0.2 ml. trypsinised or sensitised red blood cells were added to 0.2 ml. platelet rich versene plasma. The mixture was centrifuged at 3600 g for 5 minutes and gently resuspended by tapping.

The presence or absence of platelet or mixed platelet

red cell agglutination was observed in each tube.

The results are recorded in Table XXXVII.

Experiment 36

Purpose. To define the incidence of positive platelet agglutination tests in normals.

Method

Ten normal human donors, of known red blood cell group structure, each donated venous blood. This was allowed to clot in glass tubes at 37°C and aged at 37°C overnight. On the next day a further 9.7 ml. of venous blood was removed from the same donors and transferred to siliconed centrifuge tubes containing 0.3 ml. disodium versene.

Serum and platelet suspensions were prepared from each donor as defined for the platelet agglutination test (Appendix A, p.14).

0.2 ml. of each serum was added to 0.2 ml. of each platelet suspension in a siliconed tube. In all, 100 mixtures were made so that every serum sample was matched with every platelet suspension. The mixtures were incubated for 2 hours at 37°C and the presence or absence of immune platelet agglutination observed in each of the 100 samples. The results of the first hundred test are recorded in Figure 21. The identification number of each donor can be interpreted in terms of red cell group structure by reference to Table XXXVIII.

Similar series of cross agglutination experiments were performed using similar groups of 10 normal donors selected

at random until 1000 platelet agglutination tests had been performed.

The results of these 1000 tests, involving 40 separate donors, are recorded in Figure 22.

Experiment 37

Purpose. To determine the presence of A and B antigen on platelets.

Method

Suspensions of three times washed normal platelet suspensions were prepared from blood derived from donors whose red blood cells ABO group had been determined previously.

Specific antisera of two types were available.

(a) High titre serum. Anti A and Anti B serum derived from rabbits immunised against specific Group A or B substance (supplied by Dr. M.M. Pickles)

(b) Low titre serum. Anti A and Anti B human blood grouping serum (National Blood Transfusion Service).

Both types of serum were heat treated at 56°C for 60 minutes and absorbed with barium sulphate powder before addition to platelet suspensions (see Appendix A, p.4).

0.2 ml. undiluted specific anti-A and anti-B sera were added to 0.2 ml. of each washed platelet suspension and the mixtures incubated for 2 hours at 37°C.

The presence or absence of immune platelet agglutination in each sample was observed by phase contrast microscopy.

The results are recorded in Table XLI.

The titres of both the high titre and low titre anti-A and B sera were determined, after heat treatment and absorption with barium sulphate powder, by addition of 0.2 ml. samples to equal volumes of washed suspensions of washed red cells or platelets derived from donors of known ABO group.

The high titre serum was titrated by doubling dilutions of a 1/10 dilution in 0.85% saline; the low titre serum by doubling dilutions of this serum.

The results are recorded at the foot of Table XLI.

Experiment 38

Purpose. To determine whether platelets carry adsorbed A or B substance on their surface.

Method

Platelet suspensions were prepared from platelet-rich versene plasma derived from donors of known A and B red cell groups and washed repeatedly (Appendix A, p.13). After each wash 0.1 ml. of each platelet suspension was added to 0.1 ml. of high titre anti-A or anti-B serum (Experiment 37). In all, the platelets were washed ten times. The mixtures were incubated for 2 hours at 37°C and the presence or absence of platelet agglutination observed by phase contrast microscopy.

The results are recorded in Table XLI A.

Experiment 39

Purpose. To confirm the existence of ABO platelet groups by the mixed red cell platelet agglutination test (Coombs & Bedford 1955).

Method

Washed platelet suspensions were prepared as detailed in Appendix A, p.13, from donors of known ABO blood and secretor state, and the final concentration of each suspension was adjusted to give a density of approximately 500,000 platelets /cu.mm.

Anti-A and Anti-B grouping serum. Low titre (National Blood Transfusion) specific antisera.

Red Blood cells from Group O, A and B donors were washed three times in dextrose acetate saline solution (DAS) and finally resuspended to give a ½% solution.

Test

6 drops of both antisera were added to 6 drops of each platelet suspension and the mixtures incubated for 1 hour at room temperature. The platelets were separated by centrifuging at 2800 g for 10 minutes and, the supernatant having been removed, washed twice in DAS. The platelets were finally resuspended in 4 drops DAS.

1 drop of sensitised platelets was added to 1 drop of samples of ½% suspensions of Group O, A and B cells in DAS.

The mixtures were spun at 2800 g for 2 minutes, resuspended and observed by phase contrast microscopy.

The presence or absence of mixed red cell platelet agglutination was recorded and the results are presented in Table XLII.

Experiment 40

Purpose. To detect the presence of Forssman antigen in platelets.

Method

Washed platelet suspensions were prepared as in Experiment 38.

Specific Forssman antiserum was prepared in rabbits.

Washed suspensions of sheep red blood cells were injected as 1 ml. amounts of packed red cells, both intravenously and intraperitoneally, at weekly intervals for 3 weeks. 1 week after the last injection the rabbit was bled and serum separated. One sample of serum was absorbed with an equal volume of a suspension of macerated guinea pig kidney for 1 hour at 37°C to remove specific Forssman antibody.

Titre of antiserum (unabsorbed) again sheep red cells - 1/1024.

Titre of antiserum (absorbed) against sheep red cells - 1/32

The antiserum, both absorbed and unabsorbed, was used after diluting 1/64 to exclude the non-specific heterophil agglutinin.

Red blood cell suspensions.

A thrice washed suspension of sheep red blood cells was resuspended finally in DAS to give a ½% suspension. A control suspension of human Group O red blood cells was also prepared as in Experiment 39.

Test

6 drops of each platelet suspension were added to 6 drops samples of both unabsorbed and absorbed antiserum. The mixtures were incubated for 1 hour at 37°C.

The platelets were separated, washed and resuspended in DAS as in Experiment 39.

1 drop sensitised platelets was added to a ½% suspension of either sheep or human red blood cells. The mixtures were spun at 2800 g for 2 minutes, resuspended and the presence or absence of mixed red cell-platelet agglutination observed by phase contrast microscopy.

The results are recorded in Table XLIII.

Experiment 41

Purpose. To decide whether human platelets carried the Rhesus antigen.

Method

Washed platelet suspensions were prepared (Appendix A, p.13) from known Group Rhesus positive and negative donors as in Experiment 38.

Specific Anti-Rhesus Antiserum (Incomplete Anti-D). Supplied by Dr. M.M. Pickles.

Sensitised Red Cell Suspensions. Red blood cell suspensions were made from both Rhesus positive and negative donors and washed three times in DAS. The cells were sensitised as described in Appendix A, p.24, and again washed three times in DAS.

Anti-human Globulin Rabbit Serum was supplied by

Dr. M.M. Pickles and used in a dilution of 1/10.

Test

6 drops of each platelet suspension was added to 6 drops incomplete anti-Rhesus (snti-D) antiserum. The mixtures were incubated at 37°C for 1 hour.

6 drops of each red cell suspension were similarly sensitised with incomplete anti-D antisera. Both platelets and red cells were separated from the antisera and washed three times in DAS.

1 drop of sensitised platelets was added to 1 drop sensitised red cells together with 1 drop anti-human globulin serum.

The mixtures were incubated at 37°C for 30 minutes before centrifuging at 2800 g for 2 minutes. The red cell-platelet mixture was resuspended and the presence or absence of mixed red cell-platelet agglutination observed.

The results are recorded in Table XLIV.

Experiment 42

Purpose. To study the effect of Group specific anti-human platelet rabbit serum on normal platelet suspensions.

Method

Two rabbits were given injections of thrice washed human platelet suspensions prepared from donors whose theoretical platelet group had been provisionally determined (Table XLVI). Three intravenous and intraperitoneal thrice-injections of 1 ml./washed human platelets were given at

weekly intervals. 10 days after the last injection the rabbits were bled and their sera separated for testing. In parallel sera were prepared from normal rabbits.

Both antibody containing and normal sera were treated as defined in the platelet agglutination test (Appendix A, p.14).

Washed suspensions of platelets were prepared from normal human donors of known theoretical platelet group structure (Appendix A, p.13). 0.2 ml. test and normal sera were added to 0.2 ml. of each platelet suspension and the mixtures incubated for 30 minutes at 37°C. The presence or absence of platelet agglutination was observed by phase contrast microscopy.

The results are recorded in Table XLVII.

Experiment 43

Purpose. To determine the physical properties of the 'platelet agglutinating factor'.

Method

Serum, containing an apparent specific platelet agglutinin in high titre, was prepared from 50 ml. blood derived from a case of idiopathic thrombocytopenia and allowed to age at 37°C for 18 hours.

This serum was treated in various ways or exposed to varying additions (Table LIV), and then retested for its ability to produce typical immune agglutination in normal platelets. Each sample, irrespective of the treatment that it had been given, was exposed to heat (56°C) and barium sulphate absorption as in the platelet agglutination test (Appendix A, p.). 0.2 ml. of the treated serum was

added to a suspension of thrice washed normal platelets in DAS and incubated at 37°C for 2 hours.

The presence or absence of immune platelet agglutination was recorded in Table LIV.

Heat treated serum

1 ml. of serum was heated at 65°C for 15 minutes.

Dialysed serum

5 ml. of test serum was dialysed against 0.85% saline for 18 hours at 4°C.

Platelet absorbed serum

0.5 ml. serum, previously heated at 56°C for 60 minutes and absorbed with barium sulphate powder, was added to 0.5 ml. of packed, thrice washed, normal platelets and the platelets resuspended in this serum. The mixture was incubated at 37°C for 2 hours and the platelets separated by centrifugation at 4200 g for 30 minutes. The supernatant serum was retested for its ability to induce typical immune agglutination in washed platelets.

Red cell absorbed serum

The procedure detailed above was repeated using 0.5 ml. packed, thrice washed red blood cells.

Ammonium sulphate fractionation

Three fractions of serum were prepared by ammonium sulphate precipitation as described in Experiment 14.

Euglobulin fraction was prepared as described in Experiment 14.

Other procedures are detailed in Table LIV.

Experiment 4.

See Appendix C, Case 65, and Chapter XII p. 165.

Preparation of Patient's Human Albumen

36 ml. of the patient's venous blood was added to 4 ml. 3.8% sodium citrate.

30 ml. high spun plasma was obtained and to this was added 30 ml. saturated ammonium sulphate (to give a 50% saturated solution).

This was allowed to stand for 1 hour at 0-4°C, and the precipitate was separated by centrifuging at 6,800 g for 15 minutes at 15°C.

The supernatant was removed and to this was added 10.3 gms. ammonium sulphate to give a saturated solution according to the formula.

$$\frac{V \times 25.7}{100} + X = (V + \frac{X}{2}) \times \frac{51.4}{100}$$

V = volume supernatant (30 ml.)

X = weight of ammonium sulphate to be added.

This solution was allowed to stand for 1 hour at 0-4°C and spun at 6800 g for 20 minutes at 4°C. The precipitate floated to the top and the infranatant was carefully removed.

The precipitate was dissolved in 0.85% saline and dialysed against 0.85% at 4°C for 18 hours.

The pH of the dialysed fluid was adjusted to pH 7.0 using 1% ammonium carbonate.

The concentration of albumen was found to be 0.78 gm% (Dr. E. Bidwell).

In vitro platelet agglutination tests and clot retraction inhibition tests were performed as for drug sensitivity tests (Appendix A, p. 16) using test and control albumen solutions in the above concentration instead of solutions of drugs.

Solutions of Human Albumen (Lister) 0.78%

" " Human Albumen (American) 0.78%

" " Bovine Albumen 0.78%

" " M.D. Albumen 0.78%

were used in each test system.

The results are described in the case history (Case 65) (Appendix C).

APPENDIX C.

SUMMARIES OF CLINICAL AND LABORATORY FINDINGS IN 73 CASES OF THROMBOCYTOPENIC PURPURA AND RELATED CONDITIONS.

All these cases have been interviewed by myself and in many instances histories were obtained and laboratory investigations were carried out personally. On those cases under the care of Professor L.J. Witts, Laboratory investigations (Haemoglobin, bleeding time (Duke 1912) Capillary Fragility, Platelet Count (Dameshek 1932) and Sternal biopsy) marrow/were performed in the laboratories of his unit.

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1. D.S.(Female) aged 57. This lady had bruised easily all her life but 2 months prior to admission to hospital she developed a sore throat. This was associated with an increased tendency to bruise. It was not apparently spontaneous but followed trivial trauma. She was found to be thrombocytopenic 10,000/cu.mm. and was placed on cortisone and then prednisolone therapy as an out patient, but her bruising persisted.

On admission to hospital physical examination revealed numerous resolving bruises over chest, arms and legs. There was no splenomegaly.

Laboratory Investigations. Hb. 104% - 15.4 gms.%. Platelets 10,000/cu.mm. Capillary fragility was increased. Platelet agglutination test - negative. Marrow biopsy - megakaryocytes plentiful. Further steroid therapy

produced no improvement and was discontinued.

This patient was discharged under observation and splenectomy may be performed shortly.

Previous Blood Transfusion - nil. Previous Pregnancy - nil.

Summary. Acute idiopathic thrombocytopenic purpura in a female aged 57.

2. G.F.(Female) aged 22. This girl was first seen in 1949 when she had an acute agranulocytosis, WBC 400/cu.mm. thrombocytopenia (19,100/cu.mm.) and mild anaemia (Hb. 8gms%) No cause was discovered for this acute episode. She responded to parenteral penicillin therapy and, following blood transfusion, there was a spontaneous return of her blood picture to normal. In 1954 she was delivered of a normal male child and there were no complications, but she developed menorrhagia following this confinement. In January 1955 she suddenly started to bruise spontaneously and had mild epistaxes. Physical examination revealed that the spleen was enlarged. Again she was anaemic and thrombocytopenic but her white count was normal. The Bone marrow was hypoplastic but megakaryocytes were present. The bleeding time and capillary fragility were abnormal. Cortisone therapy produced a clinical improvement and the platelet count returned slowly to normal. This remission lasted 2 months but she again relapsed with acute thrombocytopenia and spontaneous bruising and purpura. Splenectomy was performed in June 1956 with a dramatic return of platelet count to normal and complete clinical

recovery.

She is now fit and well with a normal blood picture (December 1956).

Platelet Agglutination Tests - positive Jan 1955. No titre recorded. Negative Oct 1956(Post splenectomy).

Previous Blood Transfusions - 2. Previous Pregnancy - 1.

Summary. Chronic idiopathic thrombocytopenia presenting originally as a case of Hypersplenism with thrombocytopenia.

3. K.O.(Male) 14. This boy suffered from recurrent episodes of spontaneous purpura, epistaxes and bruising since the age of 3. Repeated examinations have always revealed a thrombocytopenia 10-30,000/cu.mm., a prolonged bleeding time - usually 15' (Ivy) and abnormal capillary fragility. The spleen was never been enlarged.

Marrow biopsy showed numerous megakaryocytes in an active normoblastic marrow. Platelet agglutination has been positive ($\frac{1}{8}$) on the two occasions it has been tested.

Splenectomy has been refused by the Boy's parents and steroid therapy has not been given.

Previous Transfusions - nil.

Summary. Chronic idiopathic thrombocytopenia in a boy aged 14.

4. B.F.(Female) aged 28. This young lady was admitted to hospital in January 1955 because of a sudden

onset of purpura and bruising of 5 days duration. Physical examination showed that the purpura was confined to her legs and chest and there were occasional bruises on her legs. The spleen was not enlarged and no other abnormality was detected. She had been taking dextro-amphetamine because of increasing obesity but had had none for 1 month prior to the onset of the purpura.

Laboratory investigations. Hb. 88% 13.0 gms%. White cell count 5,600/cu.mm. Platelets were too few to count. The bleeding time was prolonged (15' + Ivy) and the capillary fragility was abnormal. Platelet agglutination test was negative. Sternal marrow biopsy showed a hyperplastic marrow with abundant megakaryocytes.

Cortisone therapy produced an immediate remission with a rise in the platelet count to normal level with 10 days. (Platelets 310,000/cu.mm.). This therapy was maintained for 2 months and, as the remission had been maintained, the dose was tailed off and therapy stopped. There had been no occurrence of purpura or bruising 18 months later.

Previous Blood Transfusion - nil. Previous Pregnancy - nil.

Diagnosis. Acute thrombocytopenic purpura in a girl aged 28.

5. R.F. (Female) 52. This woman has had episodes of spontaneous bruising for several years. Because of gross obesity she has been attending hospital and it was observed in 1953 that there was a purpuric rash involving

one shoulder. Physical examination revealed no abnormality apart from gross obesity and splenomegaly.

Laboratory investigation revealed that she was then thrombocytopenic 40,000/cu.mm., the bleeding time being 15' + (Ivy) and capillary fragility was increased.

She has been seen at frequent intervals since this episode, but although she still continued to bruise, no episodes of spontaneous purpura occurred. Her platelet count fluctuated, being normal at one time, 200,000 - 500,000, with normal bleeding time and tourniquet test and markedly abnormal the next (too few to count).

In January 1956 she was seen because of a recurrence of her purpura. Platelets 25,000/cu.mm. B.T. 15' + (Ivy), capillary fragility - abnormal. Platelet agglutination test was positive

She was placed on the waiting list for steroid therapy but to date has refused to come into hospital.

Previous Blood Transfusions - nil. Previous Pregnancies - 2.

Summary. Recurrent chronic thrombocytopenia in a female aged 52.

6. P.V.(Male) 30. This man was admitted to hospital following the sudden onset of widespread purpura, epistaxes, and gum haemorrhages 2 days previously. For the previous 2 years he had taken "Anadine" tablets for recurrent headaches. (Anadine tab = Aspirin gr. 3, Phenacetin gr. 3, Caffeine gr. ¼, Quinine gr. 1/12.) Immediately prior to the onset of spontaneous haemorrhages he took 8 tablets

but had had none for the 12 days prior to admission.

Physical examination revealed a widespread petechial rash on the trunk and legs and there were several blood blisters in the mouth. The spleen was not palpable.

Laboratory investigations. Hb. 114%. 16.9 gm% White cell count 10,400/cu.mm. Platelets - too few to count. Bleeding time 15' + (Duke). Capillary fragility - not recorded. Marrow Biopsy - Megakaryocytes were plentiful. Platelet agglutination test - negative. In vitro tests for aspirin, phenacetin, caffeine and quinine sensitivity were negative. (Clot Retraction and Modified Platelet agglutination test see Appendix A p.) No skin tests were performed and no trial dose of quinine was given.

Cortisone therapy was given and there was immediate improvement in his haemostatic mechanism in that his bleeding time returned to normal. His platelet count did not rise at first but slowly climbed to normal in 2 months. The improvement was maintained following withdrawal of the steroid therapy.

Previous Blood Transfusions - nil.

Summary. Acute thrombocytopenic purpura in a male aged 30. This may have been provoked by quinine sulphate but in vitro laboratory tests did not confirm this.

7. D.S.(Female) aged 60. This woman had been dyspnoeic for many years and during the past 2 years had

complained of retro-sternal pain when she stooped or lay flat. 7 months before admission she developed a black eye spontaneously. This resolved but 2 months later she had a spontaneous nose bleed and bruises appeared on her arms.

Laboratory investigation at this time revealed a marked thrombocytopenia 9,000/cu.mm. and a bleeding time of 25 minutes (Horton General Hospital, Banbury).

1 month later she had a further nose bleed and was admitted to hospital for investigation. She had been taking no drugs over this period. Physical examination revealed an obese old woman with purpura over the chest and multiple bruises of her arms and legs. Two sub-mucosal haemorrhages were present on the palate. The spleen was not palpable. No other abnormality was discovered.

Laboratory Investigations. Hb. 78%. 11.5gm%. White cell count 5,000/cu.mm. Platelets 37,000/cu.mm. Bleeding time 11'30" (Duke).

Coombs test negative. Platelet agglutination test - positive to a titre of 1/4. X-ray examination showed the spleen was enlarged X 4 normal. Marrow biopsy showed that megakaryocytes were present. No other abnormality was detected.

In view of her history no therapy was given. 3 months later her purpura and bruising still persisted and had become more severe. Splenectomy was advised but has not been performed to date.

Previous Blood Transfusions - nil. Previous

Pregnancies + ~~(number unknown).~~

Summary. Chronic thrombocytopenic purpura in a female aged 60.

8. I.B.(Female) aged 59. This lady had a radium menopause at the age of 44 for menorrhagia. She subsequently was well until the age of 58 when she began to feel vaguely unwell. During the 6 months prior to admission she developed an increasing anaemia and had bruised excessively. This culminated in the development of a purpuric rash on her limbs. She had taken no drugs apart from barbiturates.

On admission physical examination showed that she was normal apart from anaemia, Hb. 31%, 4.6 g.% with marked thrombocytopenia (7,000/cu.mm) and an unexplained reticulocytosis 18.2%. The peripheral blood and marrow showed marked iron deficiency changes. There was increased red cell activity in the marrow and Megakaryocytes were frequent. The changes were those of an haemolytic anaemia with thrombocytopenia. All investigations to determine the aetiology of the haemolytic process did not reveal any specific cause but the platelet agglutination test was strongly positive to a titre of 1/32.

Prednisone therapy induced a remission in her thrombocytopenia but she relapsed immediately it was stopped. Splenectomy was performed with immediate clinical and haematological improvement and on discharge 2 weeks after splenectomy her platelet count was 858,000/cu.mm. and her Hb. 92% (13.5 gm). Reticulocytes 0.6%. Platelet

agglutination test - post splenectomy + 1/16. (1 week).

Diagnosis. Chronic Idiopathic thrombocytopenic purpura with associated haemolytic anaemia responding to splenectomy.

Previous Blood Transfusion + 1. Previous Pregnancy +

9. L.S.(Male) aged 52. This man was originally seen in 1953 with enlarged cervical lymph nodes at first presumed to be tuberculous.

In 1954 the lymph nodes in the left side of his neck were removed surgically and the histological picture was suggestive of Hodgkins disease (lymphadenoma). Radiotherapy produced an immediate regression in the size of his lymph nodes.

6 months later he was admitted to hospital with a generalised pancytopenia. Physical examination revealed enlarged lymph nodes in the cervical and axillary regions. Both the liver and spleen were enlarged. No other significant abnormality was discovered. There was a persistent pyrexia.

Laboratory investigations. Hb. 48% 7.1 gms.% White Blood cells 1,500/cu.mm.(Neutrophils 1080/cu.mm) Platelets 38,000/cu.mm. E.S.R. 12 mm/hour. Sternal marrow biopsy revealed a hypoplastic normoblastic marrow. The presence or absence of megakaryocytes was not recorded. Tubercle bacilli were isolated from the urine which contained occasional pus cells.

Plasma proteins. Total 5.0 gms.%. Albumen 2.9 gms%.

Streptomycin and P.A.S. therapy were given with a good clinical response and the patient became afebrile but the pancytopenia persisted.

When seen in October, 1956, Platelet agglutination test was negative.

Previous Blood Transfusion + 1

Summary. Pancytopenia with thrombocytopenia associated with splenomegaly due to lymphadenoma.

Addendum.

Splenectomy was performed in February 1956 with a slow but definite response both clinically and haematologically. Platelets 407,000/cu.mm. 10 months later he slowly relapsed and his pancytopenia was again present (Platelets 5,000/cu.mm). Platelet agglutination test at this time was negative. Histological examination of the Spleen did not decide the exact pathology in this man; the changes were not typical of Lymphadenoma.

10. W.W.(Male) aged 35. This man was admitted to hospital as an emergency following a haematemesis and melaena. Previous history was negative apart from beri-beri, amoebic dysentry and malaria while a Japanese P.O.W.

On admission he did not appear to have lost much blood. His blood pressure was normal and his haemoglobin was 80% (11.8 gms%). Haematocrit 31%. He was confused and maniacal but no localising C.N.S. signs were elicited. Laboratory investigation showed marked thrombocytopenia 64,000/cu.mm. although the bleeding time and capillary fragility were normal. All tests of coagulation function

were normal. Bone marrow biopsy revealed a hyperplastic normoblastic marrow with abundant megakaryocytes. X-ray examination could not exclude the presence of a duodenal ulcer, but no definite ulcer was demonstrated. Platelet agglutination test was positive 1/4. He continued to have occult blood in the stools and splenomegaly developed.

The thrombocytopenia persisted but he did not develop any purpura or bruising. He continued, however, to have occult blood in his stools. 6 months later he was readmitted for dental treatment and his thrombocytopenia was still present (Platelets 12,000/cu.mm.) The bleeding time was prolonged 11' (Ivy) and capillary fragility was abnormal. Again the platelet agglutination test was positive 1/4.

1 year after the first episode he had had no recurrence of his gastro-intestinal bleeding nor had he had any other haemorrhagic episode. His thrombocytopenia and splenomegally still persisted. At no time had this man any specific therapy for his thrombocytopenia.

Previous Blood Transfusion + 1.

Summary. Chronic Idiopathic Thrombocytopenia in a male aged 35.

11. N.C.(Female) aged 24. The woman has suffered from purpura and spontaneous bruising since the age of 8. The attacks have been intermittent and in between the attacks she has been perfectly normal apart from menorrhagia since the menarche. Her present attack was of 5 months duration and was associated with widespread

purpura and bruising.

Physical examination revealed no abnormality apart from the purpura.

Laboratory Investigations. There was an iron deficiency anaemia. Hb. 68% (10.1 gms%). Platelet count was 14,000/cu.mm. and the bleeding time was prolonged 15' +(Ivy). Clotting time and prothrombin time (Quick) was normal. Coomb's test negative(on red blood cells). Sternal marrow showed megakaryocytic hyperplasia. Platelet agglutination test was positive to a titre of $\frac{1}{4}$.

As she wished to start a family, splenectomy was advised but has not yet been carried out.

No Previous Blood Transfusions. No previous pregnancies.

Diagnosis. Recurrent chronic idiopathic thrombocytopenia.

12. S.A.(Female) aged 10. This child had had persistent thrombocytopenia with an abnormal tendency to bruise for 2 years and there was a severe haemorrhage following the extraction of one tooth. There were no epistaxes, haematuria or evidence of gastro-intestinal haemorrhage.

When seen in the Radcliffe Infirmary in April 1956 no physical abnormality was detected apart from bruises on the right leg. The spleen was not enlarged.

Her bleeding time was prolonged 15' + (Ivy) and her capillary fragility was increased. The platelets were too few to count. In view of her approaching menarche,

splenectomy was performed with immediate clinical and haematological return to normal.

Platelet agglutination tests both pre and post splenectomy were negative.

No Previous Blood Transfusions.

Diagnosis. Chronic idiopathic thrombocytopenia in a child aged 10.

13. E.R.(Female) aged 61. In 1944 this woman noticed that numerous spontaneous bruises were appearing on the legs and trunk and she had recurrent epistaxes. These were found to be associated with thrombocytopenia which responded dramatically to immediate splenectomy(Birmingham). In 1953 there was an isolated short-lived recurrence of the sponteneous bruising and purpura together with mild epistaxes. Laboratory tests showed she was again thrombocytopenic.

2 years later there was a further recurrence of the bruising, purpura and epistaxes. She had been taking no drugs prior to this episode. She was admitted to the Radcliffe Infirmary, Oxford where physical examination revealed widespread purpura and bruising on the legs and there were several blood blisters in her mouth. No other abnormality was detected. The spleen was not enlarged.

Laboratory investigations. Hb. 100%. 14.8 gms. White cell count 12,800/cu.mm. Platelets 11,000/cu.mm. Bone marrow biopsy:- Hyperplastic normoblastic marrow with abundant megakaryocytes. Coomb's Test on red blood cells - negative. Platelet agglutination test - negative.

Cortisone therapy produced a dramatic remission within 1 week (platelets 334,000/cu.mm) This was maintained following withdrawal of the drug.

One previous blood transfusion. One previous pregnancy.

Summary. Recurrent chronic thrombocytopenic purpura in a female aged 61.

14. A.M.L (Male) aged 10. This boy was first seen in July 1955 with a mild attack of mumps, associated with widespread subcutaneous bruising, purpura and epistaxes, which had been present for 1 week. He had been known to bruise easily, following trauma, since the age of 5 but apart from very small nocturnal epistaxes he had not had any other evidence of spontaneous haemorrhage.

Physical examination showed widespread confluent bruises over both legs and numerous petechial haemorrhages on the trunk. Both parotid glands were enlarged and slightly tender and there appeared to be a slight ooze from the left tonsil. There was no splenomegaly.

Laboratory investigations. Hb. 102%(15.1 gms%) White cell count 6,800/cu.mm. Platelets were too few to count. The bleeding time was 15' + (Ivy) and capillary fragility was abnormal. Clot retraction was nil after 1 hour at 37°. Platelet agglutination test was positive to a titre of $\frac{1}{2}$. The purpura and bruising persisted together with thrombocytopenia following clinical recovery from mumps and in view of his pending entry into boarding school an immediate splenectomy was performed. This

produced a dramatic clinical improvement and the platelet count, bleeding time, capillary fragility and clot retraction all became normal within 25 days. This improvement persisted and he was discharged . 3 months later his clinical improvement had been maintained but there was a mild thrombocytopenia (Platelets 90,000/cu.mm). The platelet agglutination test was still positive at this time.

No previous blood transfusions.

Summary. Acute thrombocytopenic purpura in a boy aged 10.

15. E.P.(Female) aged 70. For the past year this woman had had symptoms of anaemia, associated with spontaneous bruising, purpura and recurrent epistaxes. Iron therapy had produced no response. Blood transfusion was given prior to her admission to Radcliffe Infirmary, Oxford.

On admission there was widespread purpura over her arms, legs and front of chest and there were multiple haemorrhagic blisters on the inner aspect of the cheeks and lips. There was marked pallor and koilonychia. The liver was enlarged but the spleen was not palpable.

Laboratory investigations. Hb. 54%/8.0 gms% Reticulocytes 6.2%. White cell count 1,900 (Neutrophils 500/cu.mm) Bleeding time 15' +(Ivy). Platelets were too few to count. Direct Coomb's Test on red blood cells - negative. Platelet agglutination test - negative. Sternal marrow was cellular and normoblastic; myelopoesis was depressed and no megakaryocytes were seen. Stools persistently contained

occult blood. Steroid therapy (Prednisolone) produced a rise in the number of white cells but the thrombocytopenia and haemorrhagic state persisted. (Platelet 8,000/cu.mm. B.T. 15' +.) Platelet transfusion improved the haemorrhagic state but did not produce any rise in the platelet count.

Splenectomy was performed which produced an immediate improvement in her haemorrhagic state. The bleeding time fell to 8' and the spontaneous purpura and bruising ceased. The platelet count rose to 81,000/cu.mm. but fell to levels varying between 17 & 38,000/cu.mm.

Unexplained bilateral supra-clavicular swellings appeared soon after her admission. These were originally thought to indicate a mediastinal haemorrhage but they did regress with the improvement in her condition following splenectomy.

One previous blood transfusion. Three previous pregnancies.

Summary. Thrombocytopenic purpura due to a probable idiopathic hypersplenism in a woman aged 70.

16. A.C.(Male) aged 9. This boy developed acute purpura following a febrile illness of an unspecified nature. During the febrile phase he was given 3 'John Bell' capsules which contain quinine, camphor, aspirin, phenacetin and phenolphthalein. The purpura persisted and, when he was seen in Oxford 2 months later, he was markedly thrombocytopenic (10,000/cu.mm.) in spite of having received Prednisone therapy for 1 month.

Laboratory investigation showed that his bleeding

time and capillary fragility were normal, but that clot retraction was abnormal (35% after 1 hrs. incubation at 37° C.) Physical examination revealed that the spleen was enlarged. Splenectomy was advised if a further period of 'Prednisone' therapy produced no improvement.

When seen after a further 2 weeks' therapy his condition had worsened. The bleeding time was 15' +, capillary fragility was abnormal and the platelets were too few to count. Direct platelet agglutination test on both visits were negative.

In view of the purpura apparently following the administration of drugs, he was tested for drug sensitivity 1 month after the drug had been withdrawn. There was no evidence at that time of sensitivity to quinine, camphor, aspirin or phenacetin as measured by platelet agglutination tests and clot retractions (Ackroyd 1950) (See Appendix Ap). Steroid therapy was stopped gradually and before splenectomy could be performed he underwent a spontaneous remission with complete clinical recovery. The platelet count and all tests of haemostatic function returned to normal.

No previous blood transfusions.

Summary. Acute thrombocytopenic purpura in a boy aged 9.

17. B.P.H.(Male) aged 10. This boy had suffered from spontaneous bruising and the occasional nose bleed for the past year and was admitted to Stoke Mandeville Hospital following a sudden exacerbation of this bruising tendency and the development of a widespread petechial rash.

Physical examination revealed widespread purpura and numerous bruises. The spleen was enlarged and the platelet count was 60,000/cu.mm. He also at this time had colicky central abdominal pain and passed altered blood in his stools. 2 monthslater he was readmitted following a fall, bleeding continuously from nose and mouth. There was also gross haematuria. The bleeding persisted and Prednisolone therapy and repeated blood transfusions had no beneficial effect.

Laboratory Investigation showed that his blood platelet count was 36,000/cu.mm. Bone marrow biopsy showed that numerous megakaryocytes were present. Splenectomy was performed but the bleeding continued and the platelet count did not rise above 80,000/cu.mm. Further Prednisolone therapy produced no improvement and he was transferred to the Radcliffe Infirmary, Oxford.

On admission, Physical examination showed that there was widespread purpura and numerous bruises were present over the trunk and lower limbs. A large blood clot was present in the nasopharynx.

Laboratory Investigations. Hb. 105%. White cell count 13,000/cu.mm. Platelets - too few to count. Capillary fragility abnormal. Tests of coagulation function showed no other defect apart from thrombocytopenia. Platelet agglutination test negative. The platelets remained too few to count but large doses of Prednisolone reduced his bleeding time and capillary fragility to within normal limits. Spontaneous bruising also ceased. In view of his poor response to steroid therapy the drug

was gradually withdrawn without any worsening of his clinical condition. The platelet count remained persistently low. No follow up notes are available.

One Previous blood transfusion.

Summary. Acute thrombocytopenic purpura in boy aged 10.

18. R.L.(Male) aged 3½. This little boy was seen as an outpatient with widespread purpura and bruising of 5 days duration. The onset had been associated with a febrile episode and stiff knee joints.

Physical examination revealed widespread purpura and many bruises. There was no splenomegaly.

Laboratory examination. Hb. 92% 13.6 Gm. White blood cells 7,000/cu.mm. Platelets 10,000/cu.mm. Bleeding time 14' (Ivy). Capillary Fragility abnormal. Platelet agglutination test - negative. He was sent home to bed and when seen 2 weeks later the purpura and bruises were disappearing and all laboratory investigations had returned to normal. Platelets 570,000/cu.mm.

No Previous blood transfusion.

Summary. Acute thrombocytopenic purpura in a boy aged 3 years with spontaneous recovery.

19. J.W.(Male) aged 48. This man was admitted to hospital for investigation of widespread purpura of sudden onset.

For the past 2½ years he had bruised easily and during the past 2 years had had recurrent epistaxes and

blood blisters in the mouth.

3 years before he had Herpes Zoster of the intercostal nerves. This was treated by Chlorampenicol (12 gms. in 2 weeks). Otherwise he had been a perfectly fit man.

Physical examination revealed widespread bruises of arms and legs together with widespread purpura of legs, arms, trunk and lips. The spleen was not enlarged.

Laboratory Investigations. Hb. 112%/16.5 Gm/100 ml. W.B.C. 10,800/cu.mm. Platelets too few to count. Bleeding time (Ivy) 15' +, Clotting Time (Lee & White) 7 mins. Coombs Test on red blood cells - negative. Capillary Fragility - markedly increased. Marrow Biopsy - A cellular normoblastic marrow. Megakaryocytes easily found. Abdominal x-ray revealed mild splenomegaly: Platelet agglutination test was positive to a titre of 1/8.

Cortisone therapy produced no shortening of his bleeding tendency, or any rise in the platelet count. His spontaneous purpura and epistaxes continued and became gradually more severe. He was readmitted following the sudden onset of a left facial palsy of upper motor neurone type and severe sciatica. There was widespread purpura and numerous sub-mucosal haemorrhages were present inside the mouth. Again his bleeding time was prolonged(12' - Duke) and the platelets were too few to count. Platelet agglutination test was again positive 1/4.

In view of the severity of his symptoms splenectomy was performed. There was a sudden copious secondary haemorrhage post-operatively and the patient

died in spite of intensive transfusion of fresh blood.

At post-mortem the peritoneal cavity was filled with blood and blood clot which had come apparently from the splenic pedicle. There was also evidence of an old cerebral haemorrhage involving the left temporal lobe.

No previous blood transfusions.

Summary. Chronic thrombocytopenic purpura in a man aged 48.

20. D.S.(Female) aged 31. This woman had had a bleeding tendency all her life. Severe haemorrhage followed tooth extraction and menorrhagia has been present since the menarche. Her sister died at the age of 3 following tonsillectomy.

She was admitted to hospital for investigation prior to multiple tooth extractions. Physical examination revealed that there was purpura over the knees and elbows and that the spleen was enlarged.

Laboratory Investigations showed that she was anaemic, (Hb. 7.0 gms) with marked iron deficiency changes. Bleeding time was prolonged 15' (Ivy) and there was a thrombocytopenia (32,000/cu.mm.) The platelets present showed numerous giant forms. In spite of the thrombocytopenia, clot retraction, prothrombin consumption and thromboplastin generation test (Patients platelets) were normal. There the platelets in spite of being decreased in number showed normal function. These unusual findings together with positive family history suggested that this might be a case of a congenital functional platelet defect rather than a typical case of chronic idiopathic thrombocytopenia.

No other members of the family were available for testing.

Platelet agglutination tests were consistently negative.

Tooth extraction was followed by severe haemorrhage but this was controlled by splints and blood transfusion.

Cortisone produced no remission.

Splenectomy was deferred in view of the strong family history.

One Previous blood transfusion. One previous pregnancy.

Diagnosis. Chronic Thrombocytopenic Purpura in a female aged 31.

21. E.B.(Female) aged 75. This old lady was admitted to hospital because of spontaneous vitreous haemorrhages in both eyes. Physical examination revealed no other abnormality.

Laboratory investigation showed that she was thrombocytopenic (50,000/cu.mm.) The bleeding time was prolonged 15' + (Ivy) and capillary fragility was increased.

In view of her age and general condition she was discharged home without further investigation or therapy.

Platelet agglutination test - negative.

Four Previous pregnancies. She had no memory of previous blood transfusion.

Diagnosis. Idiopathic Thrombocytopenia of unknown duration.

22. E.T.(Female) aged 26. This young woman was first seen at the age of 19 when she was complaining of the sudden onset of spontaneous bruising and purpura. At the same time she developed severe menorrhagia.

Physical examination revealed no abnormality apart from widespread purpura and bruising. There was no splenomegaly.

Laboratory Investigations. Hb. 74%. White cell count 2,100/cu.mm. Platelets 21,000/cu.mm. Bleeding time 39' (Ivy) Capillary fragility was increased.

Immediate splenectomy was performed but no clinical or haematological improvement followed. Bone marrow biopsy performed after splenectomy showed that the marrow was hypoplastic and no megakaryocytes could be found.

Recurrent purpura continued and menorrhagia continued. In 1951 Cortisone therapy provoked a clinical remission but the platelet count never rose above 40,000/cu.mm. Again the bone marrow biopsy showed that no megakaryocytes were present. For 4 years she was completely symptom free and during this time gave birth to a full time normal male infant. 10 months ago all her symptoms recurred.

Prednisone therapy provoked a further clinical remission but again, there was no rise in her platelet count. This remission lasted 5 months and then her purpura, bruising and menorrhagia recurred but with increased severity, necessitating admission for blood transfusion. The menorrhagia was so severe that

hysterectomy was advised but deferred to allow her to have a further pregnancy.

Laboratory Investigations. Platelet count 14,000/cu.mm. B.T. 15' + (Ivy). Capillary fragility increased. Platelet agglutination test - negative.

One previous blood transfusion. One previous pregnancy.

Summary. Chronic thrombocytopenic purpura in a female aged 26.

23. E.F. (Female) aged 42. This woman was admitted to a nursing home for hysterectomy. She had had menorrhagia and intra-menstrual loss for 5 years. There was no other history of any haemorrhagic tendency and she never had had any purpura.

Physical examination revealed no abnormality. The spleen was not enlarged.

Laboratory investigation showed a mild thrombocytopenia 109,000/cu.mm. Hb. 89% (13.2 gms%). Platelet agglutination test - positive to a tiire of $\frac{1}{2}$.

The hysterectomy was performed and there were no post-operative complications.

As this patient was a private patient no follow up laboratory investigations were performed prior to her discharge from the Nursing Home.

No previous blood transfusion. Two previous pregnancies.

Summary. Mild thrombocytopenia with menorrhagia in a female aged 42.

24. M.C.(Female) aged 51. This woman was first seen in April 1956 when she complained of easy bruising of her upper arms and legs for several weeks. Previously she had been a fit woman.

Physical examination revealed no abnormality apart from subcutaneous bruises. She was not anaemic (Hb. 88% 13.0 gms%) but there was a marked thrombocytopenia, 9,000/cu.mm. and the bleeding time was prolonged (20' + Duke) Sternal marrow showed megakaryocytic hyperplasia. Prednisone therapy produced a dramatic remission clinically and haematologically and the remission was maintained on withdrawal of the drug after 8 weeks therapy.

When seen as an out patient 5 months later she was clinically well and her platelet count was 251,000/cu.mm.

Platelet agglutination tests were negative.

No previous blood transfusions. One previous pregnancy.

Diagnosis. Acute idiopathic thrombocytopenic purpura.

25. P.B.(Male) aged 13. This boy was first seen in 1953 when he complained of increasing lassitude, breathlessness and an abnormal tendency to bruise spontaneously. This tendency was accompanied by severe recurrent and spontaneous epistaxes.

Physical examination revealed an enlarged spleen and an apical systolic heart murmur. There was fine purpura of the ankles, chest and buttocks.

Laboratory Investigations:- He was anaemic

there was a further recurrence of purpura associated with clinical mumps; the platelets were on this occasion too few to count. Again there was a spontaneous remission. In 1955, a further recurrence of purpura, associated with epistaxes and haematuria and haematemesis necessitated her admission to hospital. Again the platelets were too few to count. Cortisone therapy was given but after 3 days there was a fresh haematemesis associated with a convulsion. As there was no improvement in her platelet count and the bleeding time remained prolonged, Splenectomy was performed although the spleen was normal in size. A Platelet Agglutination Test performed just prior to splenectomy was positive to a titre $\frac{1}{8}$.

Clinically she improved following the operation but the platelet count did not rise.

The purpura, bruising and epistaxes recurred 2 months after the operation and the thrombocytopenia persisted (0 - 30,000/cu.mm.). Her haemorrhagic state has persisted and when last seen in September 1956 she was covered with petechial haemorrhages but was otherwise fit and well.

No bone marrow biopsy has been performed on this child to date.

One previous blood transfusion.

Summary. Recurrent Chronic Idiopathic Thrombocytopenic purpura in a child aged 6.

27. C.M.S (Male) aged 18. This young man developed a purpuric rash 1 week after he had had a sore throat.

Slight purpura and spontaneous bruising persisted in spite of Cortisone therapy for 6 weeks. He had been taking no drugs and had been perfectly fit previously. 6 months after the onset of his symptoms he was admitted to the Radcliffe Infirmary, Oxford, for investigation.

Physical examination revealed sparce purpura of the legs and there were several blood blisters in the mouth. There was no splenomegaly.

Laboratory investigations. Hb. 100% 14.8 gms%. White cell count 6,100/cu.mm. Bleeding time 11' (Duke). Platelets 41,000/cu.mm. Capillary fragility was increased. Bone marrow biopsy - megakaryocytes present. Direct Coomb's Test on red blood cells - negative. Platelet agglutination test was positive to a titre of $\frac{1}{4}$.

Splenectomy was performed with an immediate clinical and haematological response, the platelet count returning to normal levels, 512,000/cu.mm. in one week. The bleeding time was 1'30" (Duke). Platelet agglutination test performed 2 weeks after splenectomy was still positive to a titre of 1/2.

No previous blood transfusion.

Summary. Chronic thrombocytopenic purpura in a male aged 18.

28. A.K. (Male) aged 50. This man, a painter, had a sudden episode of widespread purpura for 5 days. It was confined to both legs and abdomen. It was not painful or itchy. The purpura faded leaving a faint brown staining. He had been taking no drugs in the last 6 months and was

not exposed to lead containing paints.

Physical Examination showed that apart from the fading purpura, this man was apparently healthy.

Laboratory investigation 7 days after the onset of the attack, revealed that there was mild thrombocytopenia (54,000/cu.mm) but both the bleeding time and capillary fragility were normal. Prothrombin time (Quick) was normal. No abnormal proteins were detected. Platelet agglutination test was negative.

1 month later, this man was found to be normal clinically and all laboratory investigations gave normal results. There was no recurrence of the purpura in the next 6 months. No further platelet counts performed.

No previous blood transfusion.

Summary. Acute Idiopathic Thrombocytopenic Purpura in a man aged 50.

28A. M.A. (Female) aged 57. This woman had had recurrent purpura for at least 20 years. Initially limited to her legs this purpura had spread first to the trunk, then the arms and, eventually to her face. The eruption of the purpuric haemorrhages had been preceeded usually by small indurated swellings which, on resolving, left a small area of extravasated blood.

On admission there was symmetrical purpura over legs and arms, and marked residual brown pigmentation over both legs below the knees. No other physical abnormality was noted. The spleen was not enlarged.

Laboratory Investigation. Hb. 90% (13.3 gms%)

Platelets 40,000/cu.mm. Coomb's test on red blood cells negative. Marrow biopsy was hypocellular but megakaryocytes were present in normal proportions. Platelet agglutination test was negative.

No defect of in vitro blood coagulation was detected. No abnormal proteins were found in the blood.

The platelets gradually climbed to normal levels 560,000/cu.mm. in a period of 3 weeks following the commencement of Cortisone therapy. Cortisone, was given for 9 days only before she developed acute abdominal discomfort and barium meal revealed equivocal signs of a duodenal ulcer.

Although the platelet count returned to normal, the purpura persisted. She was discharged on "Adrenoxyl" (Adrenochrome mono-semi carbazome) therapy and, when seen 3 months later, had improved clinically although the purpura still recurred at intervals.

No previous blood transfusion. Previous pregnancy.

Summary. Chronic purpura in a female aged 57 associated with a definite episode of transient thrombocytopenia.

29. G.D.(Female) aged 39. At the age of 11 this woman had had a transient haematemesis which did not recur until 1955 when she had a further severe haematemesis necessitating, blood transfusion. The stools contained altered blood. Between these episodes she had had vague symptoms of indigestion with attacks of low back pain.

These were not related to meals or improved by any medicine. A doubtful duodenal ulcer was found by x-ray. Incidentally it was discovered she had a congenital heart lesion and it was thought that this was an inter-atrial septal defect to which she was well compensated. At this time her platelet count was normal. Following transfusion there was no recurrence of her haematemesis or pain.

It was observed at follow up examination 3 months later that she had hyperextendable finger and elbow joints and it was thought that she might be a case of Ehler's Danlos Syndrome. ~~and it was suspected this might explain her symptoms.~~

Laboratory Investigation showed that she was also thrombocytopenic 72,000/cu.mm. but her bleeding time and capillary fragility were normal. Platelet agglutination test at that time was negative. On subsequent examination 2 weeks later her platelet count was 190,000/cu.mm. and the platelet agglutination test was again found to be negative.

Investigation of her family showed that her brother and her niece both appeared to be cases of Ehler's Danlos Syndrome. This confirmed the clinical diagnosis.

Comment. The only rational explanation that can be offered for this apparently transient thrombocytopenia was that her one large blood transfusion had stimulated a very weak platelet agglutinating agent, not demonstrable by in vitro testing.

One previous blood transfusion.

Summary. Transient thrombocytopenia associated

with Ehler's Danlos Syndrome in a female aged 39.

30. Mrs. B. aged 27. Baby B. (male) was born on 26th December 1954 at home, 3 weeks prematurely, and, at the age of 5 days, developed purpura of the legs. He was found to be thrombocytopenic on transfer to Gloucester Royal Infirmary and received two blood transfusions. In spite of this his platelets entirely disappeared by the 9th day. He died, aged 10 days, after numerous spontaneous haemorrhages had occurred. Coomb's Test on red blood cells was negative. Post-mortem showed widespread purpuric lesions with involvement of the brain. The mother and father of this child were referred to Oxford for investigation four months after the death of this child.

This had been the mother's third pregnancy. She had two girls aged 4 and 3. Both had been entirely normal since birth. She had been perfectly fit apart from an attack of jaundice at the age of 17. She had never bruised easily, had any purpura nor had experienced any abnormal haemorrhages. There was no family history of bleeding. She had been perfectly fit during the last pregnancy and had had no purpura. She had never required a blood transfusion. When examined 4 months later she was found to be a normal healthy woman.

Laboratory investigation:- Hb. 100% 14.8 gms%. Platelets 212,000/cu.mm. Bleeding Time 3' 45" (Ivy). Capillary fragility - normal. Platelet agglutination test showed that her serum agglutinated normal platelet suspensions, suspensions of her husband's but not her own

platelets.

The father of the child was also entirely normal and platelet agglutination tests on his serum were negative.

Summary. Neonatal thrombocytopenia causing death at 10 days in the third child of a healthy mother.

31. Mrs. D. aged 24 and Baby. Baby D male, born at Amersham General Hospital, developed purpura in the first day of life and on investigation, was found to be thrombocytopenic.

When seen on the fifth day of life the child was covered in purpura and echymoses and was slightly jaundiced. There was no splenomegaly.

Laboratory investigations. Hb. 105% (15.5 gms%) Platelet count 10,000/cu.mm. Bleeding time 10' (Heel prick). Coomb's test on red cells was negative. Platelet agglutination test done on serum derived from the baby was negative. The child continued to thrive but the platelet count remained abnormally low until 6 weeks when it returned to normal.

Mrs. D. This was this mother's first pregnancy. She had never had any miscarriages or blood transfusions.

She was perfectly normal throughout the pregnancy until at 38 weeks she developed a rash around her waist. This was not seen by a Doctor but from the patient's description this could have been purpura. It faded within 2 days and did not recur.

Physical examination revealed no abnormality and there was no apparent splenomegaly.

Laboratory investigation. Hb. 85% (12.6 gms%) Platelets 406,000/cu.mm. Bleeding time 6' (Ivy). Capillary fragility was increased. Platelet agglutination test was positive when the mother's serum was added to normal suspensions but not when added to suspensions of her own platelets. The titre was only 1/1. No opportunity presented to test the mother's serum, against her husband's platelets.

Summary. Neonatal thrombocytopenic purpura in the first-born child of an apparently healthy mother.

32. Mrs. A.S. aged 25. This woman was delivered of a full term male infant in 1952. This child, her first, developed widespread bruising and purpura over arms, legs, trunk and especially in the hands and feet at the age of 2 days. There was no previous history of illness, blood transfusions or miscarriage and at no time had the mother had any abnormal bleeding tendency or spontaneous purpura.

The baby showed no evidence of anaemia (Hb.150% 22.2 gms%) but his platelets were too few to count. Bleeding time 10'45" (Duke). Capillary fragility was normal. Coomb's test on red blood cells was negative. The mother's blood was found to contain a powerful platelet agglutinin which agglutinated her husband's platelets, and 29 out of 30 normal platelet suspensions. It did not, however, agglutinate her own platelets (Dr. R. Gillett). The purpura and bruising resolved in 4 days and the platelets returned to normal levels in 3 weeks. This baby continued to thrive as any normal child and at the age of

3 years, was a perfectly normal healthy male child. There was no recurrence of the purpura.

In 1955 Mrs. A.S. became pregnant for the second time and was admitted to hospital due to mild toxaemia. Labour was spontaneous and a full term male infant (5 lb. 11½ oz) was delivered. 2 days later the child vomitted altered blood and a few petechiae appeared on the buttocks. Platelets were again found to be too few to count. Coomb's test on the baby's red blood cells was negative. Apart from a slight ooze from the cord the child progressed normally and fed well. The platelets did not show any rise until 6 weeks after delivery (101,000/cu.mm.) and did not return to normal until 8 weeks (402,000/cu.mm.). Bleeding time and capillary fragility were found to be normal. The mother's serum again contained a platelet agglutinating factor against suspensions of her husband's platelets and 4 out of 5 suspensions of normal platelets. Her serum did not agglutinate a suspension of her own platelets. At no time during the pregnancy had the mother suffered from any bleeding tendency or purpura.

Laboratory investigation of the mother showed no evidence of thrombocytopenia (Platelets 454,000/cu.mm.). Bleeding time (Ivy) 4'. Capillary fragility - normal.

Summary . Neonatal thrombocytopenic purpura in the second child of a normal healthy mother. The first child was also affected.

33. Mrs. G. aged 28. Baby G. aged 5 days. This full time male infant started to vomit on the 5th day of life and it was noted that there was altered blood in the vomit. No other physical abnormality was discovered.

Laboratory investigation showed that the infant was thrombocytopenic (62,000/cu.mm.) There was no anaemia and white cell count showed no significant abnormality. The platelet count gradually returned to normal and was 187,000/cu.mm. one week later. (12 days). There was no further evidence of abnormal bleeding.

The child's mother (aged 28) was normal and showed no evidence of an abnormal bleeding tendency. Platelet count 336,000/cu.mm. This pregnancy, her second, was normal in every way.

Platelet Agglutination Test. The mother's serum did not agglutinate the baby's platelets, her husband's platelets or the platelets of 5 normals.

Summary. An unusual case of transient neonatal thrombocytopenia in the first-born child of a normal healthy mother.

34. Mrs. T. aged 24 and Baby T. Mrs. T. had always been perfectly fit and 3½ years ago gave birth to a normal full time male infant. In the first 2 weeks of life this boy developed a purpuric rash and had several melaena stools but in all other respects, was a normal child. 2 years later she was delivered of a 4 week premature male infant. This infant rapidly developed bruises all over the body and was transferred to hospital in Gloucester where

and this rash was considered to be toxic in nature and probably provoked by the Codein Tablets. These tablets were stopped and Histantin therapy instituted. This brought about a dramatic improvement in the rash but 1 month later she developed widespread purpura over the trunk and limbs and her original papular rash reappeared; this time with a haemorrhagic element. The drugs were stopped and she was sent to hospital for investigation 1 week later.

Physical examination showed that the purpura was fading and no fresh lesions were seen. She was a frail old woman crippled by advanced rheumatoid arthritis involving knees, hips, hands and elbows, but no other abnormality was detected.

Laboratory Investigations. Hb.: 97% (14.4 gms%) Platelets 129,000/cu.mm. Bleeding time 8' (Ivy). Capillary fragility - normal. Direct platelet agglutination tests were positive when the patients serum was added to suspensions of her own platelets and those of normals. It was found that Histantin enhanced these results. When histantin was added to the patient's platelet rich plasma and this was allowed to clot, clot retraction was inhibited completely. No such reaction took place when this drug was added to clotting platelet rich normal plasma (Appendix A p.16). No such reactions occurred when other drugs were added under similar conditions. e.g. Carbrital and Codeine. The former drug had been administered along with the Histantin. Following withdrawal of the histantin no further purpura appeared, and the platelet count returned to normal levels.

No previous blood transfusions. Previous Pregnancy.

Summary. Drug induced thrombocytopenic purpura.

36. F.H. (Male) aged 72. This patient developed in infected haematocoele as the result of surgical tapping of a right sided hydrocoele. Streptococcus faecales was isolated from the cyst and sulphamezathine therapy given. After 1 week's therapy purpura appeared on his left leg and 3 days later spread tohis right leg and thigh. There was no splenomegaly.

Laboratory investigation showed that the Hb., white count and blood film were normal. Platelet count was 80,000/cu.mm. The bleeding time was normal but the capillary fragility was increased. In vitro tests showed that the patient's serum would agglutinate suspensions of his own platelets and those of normals in the presence of Sulphamezathine, Sulphathiazole, and Sulphatriad to a titre of 1/4. No reaction took place unless these specific drugs were added. Further, these drugs were capable of inhibiting normal clot retraction when added to the patient's platelet rich plasma as it clotted. Control systems using normal serum, normal platelet rich plasma and other drugs gave entirely negative results (Appendix A.p.16).

When Sulphonamide therapy was withdrawn the petechial rash disappeared and the platelet count returned to normal two weeks later.

No previous blood transfusion.

Summary. Drug induced thrombocytopenic purpura.

37. R.H. (Female) aged 24. This young woman was admitted to hospital with an ulcerated throat associated with severe agranulocytosis, (following the treatment of clinical thryotoxicosis with methyl thiouracil for 1 year). Through a misunderstanding her white cell count had not been checked during this period.

Physical examination revealed ulcerated fauces and both tonsils were covered with exudate. The spleen was not enlarged.

Laboratory investigation. Hb. 90% (13.3 gms%) White cell count 2,000/cu.mm. (Neutrophils 350/cu.mm.) Platelets 133,000/cu.mm. Bleeding time 10' (Ivy). Capillary fragility increased.

Marrow biopsy showed a definite maturation arrest of myelopoesis at the myelocyte levels and transitional megaloblastic change in the erythroid precursors. Megakaryocytes were plentiful. Platelet agglutination test - negative. When performed with the addition of methyl thiouracil it again was negative. Leucocyte agglutination test - equivocal. Clot Retract Inhibition Tests were not performed on this patient.

Withdrawal of the drug was accompanied by a slow return of the white blood cell count and the platelet count to normal over a period of three weeks.

When seen 1 month after discharge from hospital this patient was entirely normal.

No previous blood transfusion. One previous pregnancy.

Summary. Drug induced thrombocytopenia and agranulocytosis.

38. L.N. (Female) aged 56. This patient suffered from a toxic dermatitis of hands and arms for the past 5 years. This appeared to be associated with the use of metal, floor or furniture polish.

Skin tests revealed a violent skin and systemic reaction to Johnstone's Furniture Polish, and 1 week later this patient developed spontaneous bruising and purpura of both legs below the knee.

Laboratory Investigation. Platelet count 53,000/cu.mm. Bleeding time and capillary fragility were normal. Platelet agglutination test was negative. Further testing showed that the platelet count had returned to normal one month later.

In view of the dramatic results of the skin sensitivity tests it was not considered advisable to repeat the reaction. In vitro drug sensitivity tests were not practical in view of the nature of the sensitising agent (Johnstone's Furniture Polish).

No previous blood transfusion. No previous pregnancy.

Summary. Thrombocytopenic purpura apparently due to sensitivity to Furniture Polish.

39. O.O. (Female) aged 59. This woman was originally seen in 1952 with gastro-enteritis complicating cirrhosis of the liver and splenomegaly. In March 1956 she had a severe haematemesis due to oesophageal varices. At this time she was found to be thrombocytopenic (Platelets 25,000/cu.mm.) and platelet agglutination test was positive to a titre of 1/2.

hospital with a 5 week history of congestive cardiac failure. This had been sudden in onset and it was suggested that he had had a "silent" coronary thrombosis. Electrocardiographic traces showed no abnormality. He rapidly went downhill after admission and a tentative clinical diagnosis of periarteritis nodosa was made. He suddenly, however, developed spontaneous purpura and nose bleeds.

Physical examination at this time showed no abnormality apart from scattered purpuric haemorrhages over the trunk and limbs. There was marked ankle and sacral oedema and distended jugular veins. There was no splenomegaly.

Laboratory Investigations. Hb. 66% (9.8 gms%) Platelets 15,000/cu.mm. Bleeding time 15' + (Ivy). Capillary fragility increased. Peripheral blood contained numerous immature granulocytes and occasional 'blast' cells together with occasional normoblasts. Platelet agglutination test - negative. Bone marrow biopsy revealed that normal marrow tissue was replaced by focal collections of immature cells. The appearances of the cellular clumps suggested that they were malignant tumour cells. No further investigations were performed and the patient was discharged without any primary neoplasm being located. No follow up examination has been performed to date.

No previous blood transfusions.

Summary. Secondary Thrombocytopenia due to disseminated carcinomatosis.

42 D.H.(Female) aged 56. This woman was admitted to hospital with a 6 months history of painless jaundice associated with an increased bruising tendency.

Physical examination revealed hepato and splenomegaly associated with deep jaundice.

Laboratory investigations: Serum bilirubin 6.9 - 10.5 mgms. Alkaline Phosphatase - 4 units (King Armstrong). Plasma Proteins: Total 5.8 mgms%. Albumin 1.8 gms%. Liver flocculation tests - negative. Terminally became positive. Prothrombin Ratio (Quick) 1.2 - 1.8. Hb. 48% (7.1 gms%) Retics. 5.8%. Platelets 16,000/cu.mm. The red cells were marked anisocytotic and showed many distorted forms ("fragmentation"). Coomb's test on red blood cells - negative. Platelet Agglutination Test - negative. Bone marrow biopsy revealed a hyperplastic marrow containing numerous smear cells whose exact nature was indeterminable. Red cell survival test (Asby) showed shortened survivals of normal cells when transfused into the patient.

Cortisone therapy promoted a temporary clinical improvement but had no effect on the haemolytic process or the thrombocytopenia. She rapidly relapsed and died in coma.

Post-mortem examination revealed a diffuse hepatic cirrhosis and associated with histiocytic medullary reticulosis involving the liver, spleen, lymph nodes and bone marrow.

Previous Blood Transfusion. Previous Pregnancy.

Diagnosis. Secondary Thrombocytopenia due to Histiocytic Medullary Reticulosis in a female aged 56.

43. M.L.(Female) aged 17. Admitted to hospital with a history of feeling unwell for 6 - 8 months. She complained of headaches and was febrile on admission. Temp. 103-104° F.

Physical examination. Enlarged inguinal and cervical lymph nodes. Spleen enlarged. Small bilateral pleural effusions. No other abnormality detected. Radio-chromium survival studies showed increased haemolysis.

Laboratory Investigation. Hb. 65% (9.6 gms%). W.B.C. 1400. Platelets 12,000/cu.mm. Platelet agglutination test - negative. There was a persistent Reticulocytosis. Sternal marrow. Hyperplastic normoblastic marrow showing no evidence of leukaemia. Megakaryocytes easily found. Coomb's Test on the red blood cells was negative.

Treatment. Prednisolone produced a slight improvement but she relapsed with developing jaundice. I.N.H. and Streptomycin produced remission of 10 days duration but she again relapsed, and died 1 month after admission.

Post-mortem. Acute Hodgkins disease or Histiocytic Medullary Reticulosis.

No previous blood transfusion. No previous pregnancy.

Summary. Secondary Thrombocytopenia.

44. D.F.(Female) aged 49. This woman had a long and involved illness lasting 3 years. She was first seen in 1953 when she was found to have purpura associated with thrombocytopenia and splenomegaly. 2 months later she

was found to have a spheroidal cell carcinoma of the left breast and underwent radical mastectomy.

The purpura, thrombocytopenia and splenomegaly persisted and in 1954 she commenced to have gastro - intestinal haemorrhages with melaena. She was admitted to hospital at intervals for investigation and treatment of anaemia following intestinal haemorrhages.

In September 1955 a right axillary lymph node was removed and found to be infiltrated with secondary carcinoma. Repeated x-rays and marrow biopsies failed to reveal any evidence of disseminated carcinomatosis. In February 1956 a marrow biopsy revealed a megaloblastic marrow which reverted to normoblastic maturation on Vitamin B_{12} therapy. Megakaryocytes were extremely scanty. There was, however, no improvement of her persistent anaemia or thrombocytopenia (50 - 70,000/cu.mm.)

Again in May 1956 her marrow showed megaloblastic erythropoesis but on this occasion there was no response to therapy with Vitamin B_{12} Folic acid or Crude Liver Extract.

She was now severely anaemic, (Hb. 27% 4 gms%) leucopenic (1700/cu.mm.) and thrombocytopenic (70,000/cu.mm.) Terminally she developed an unexplained eosinophilia.

She died in October 1956 and Post-mortem revealed generalised carcinomatosis with secondary deposits in bone marrow, lymph nodes and liver. The spleen was three times larger than normal. (450 gms) but was not infiltrated with tumour.

Platelet agglutination tests were persistently

negative.

Multiple Blood Transfusions. Previous Pregnancies

Diagnosis. Thrombocytopenic purpura secondary to generalised carcinomatosis arising from a primary focus in left breast.

45. J.H. (Male) Aged 53. This man was perfectly fit until 5 weeks before admission when he suddenly noticed that he was bruising excessively after trivial trauma. 2 weeks later he had a severe epistaxis and at this time he felt vaguely unwell.

On admission he had multiple subcutaneous bruises and occasional crops of petechiae on legs and feet. Apart from slight liver enlargement and axillary lymph node enlargement no other physical abnormality was found.

Laboratory Investigation revealed that he was anaemic with thrombocytopenia (19,000/cu.mm) and a rising white cell count, 15 - 33,000/cu.mm. His bleeding time was prolonged (15' +) Ivy and capillary fragility was increased. The peripheral blood film showed numerous promyelocytes and myelocytes and marrow smears showed an acute promyelocytic leukaemia. Megakaryocytes were absent. Platelet agglutination test was negative.

There was no response to Prednisolone or 'Myeleran' therapy or to blood transfusion.

He died suddenly 2 weeks after admission and post-mortem revealed a cerebral haemorrhage and tissue and marrow changes consistent with acute myeloid leukaemia.

No Previous blood transfusion.

Diagnosis. Thrombocytopenia secondary to acute myeloid leukaemia.

46. A.H. (Female) aged 57. This woman was admitted to hospital with a 1 year history of epigastric discomfort after meals. This was accompanied by anorexia. For three months there had been severe pain following meals and during the 2 weeks prior to admission she had vomited after every meal.

Laparatomy revealed generalised abdominal carcinomatosis with the primary located in the stomach. The spleen was enlarged but did not appear to be infiltrated with neoplasm.

Laboratory Investigations. Hb. 103% (15.2 gms%) White blood cells 5,900. E.S.R. 3. Platelets 95,000/cu.mm. Capillary fragility - increased. Platelet agglutination test was positive to a titre of 1/1 on two occasions.

No previous blood transfusion. Previous pregnancies 2.

Summary. Thrombocytopenia secondary to disseminated carcinomatosis in a female aged 57.

47. M.B. (Male) aged 67. This patient was admitted in fully developed congestive cardiac failure with associated peripheral gangrene of fingers and nose. There was associated cutaneous purpura of both lower limbs. He had complained of increasing dyspnoea and orthopnoea for 6 weeks prior to admission. His heart was enlarged and

there were systolic and diastolic aortic murmurs and a faint mitral diastolic murmur.

Laboratory Investigation revealed that he was thrombocytopenic (17,000/cu.mm.) As it was considered that his peripheral gangrene might have been due to recurrent platelet emboli, provoked by an immune platelet agglutinin, platelet agglutination tests were performed. These were, however, negative. His gangrenous tissue demarcated and separated but in spite of therapy he remained a state of congestive heart failure and died 4 months later after transfer to another hospital. Post-mortem revealed left ventricular hypertrophy with aortic stenosis.

No record of previous blood transfusion.

Summary. Thrombocytopenic purpura associated with congestive cardiac failure and peripheral gangrene in a male aged 67.

48. W.W. (Female) aged 40. This woman was admitted to hospital following a severe ante partum haemorrhage. It was noted that she had several cutaneous petechial haemorrhages on the trunk arms and legs. She had had two previous normal pregnancies and both children were normal.

On Physical Examination she was noted to be excessively pale and her B.P. was recorded as 80/0 .

Blood was removed for cross matching with fresh donors and it was observed this did not clot in the tube after 30 mins. Immediate laboratory investigation showed a marked fibrinogen lack, accompanied by thrombocytopenia

pregnancy was uneventful and there was no evidence of thrombocytopenia in the mother or baby.

2 Previous blood transfusions.

Summary. Thrombocytopenic purpura associated with Acute defibrination syndrome of Pregnancy.

50. J.S.(Male) aged 67. This man had been perfectly fit until 1955 when he developed a virus pneumonia from which he apparently recovered. In June 1956 he was admitted to Northampton General Hospital complaining of giddiness, headaches, breathlessness. He had had one sudden epistaxis one week prior to admission.

Physical examination revealed ecchymotic subcutaneous haemorrhages of the right hand and left arm. The spleen was enlarged.

Laboratory Investigations. Hb. 72% (10.7 gms%) White cell count 23,300/cu.mm. Reticulocytes 14%. Normal distribution. Direct Coomb's Test (Patient's red cells) - positive. Cold agglutinins 1/1. Platelets 95,000/cu.mm. Bleeding time - 3'10" (Duke). Platelet agglutination test - negative. Bone Marrow biopsy. Active cellular marrow. Megakaryocytes present.

Cortisone therapy restored his platelet count to normal levels, but his haemolytic anaemia persisted. He developed suddenly a Salmonella Typhi-murium septicaemia which necessitated cessation of steroid therapy. At no time had he had any blood transfusions. He was discharged from hospital and no follow up examination has been made to date.

Summary. Thrombocytopenic purpura associated with

an acquired haemolytic anaemia.

51. G.(Female) aged 34. This woman was first seen in 1954 when she was found to have an acute haemolytic anaemia, due to an auto & iso cold agglutinin. Direct Coomb's Test on the red blood cells was positive. A specific anti-S iso-agglutinin was present; the patient's red cell group being S-

Physical examination revealed no abnormality apart from splenomegaly. She was treated by multiple blood transfusions and cortisone without improvement.

In January 1955 as her anaemia persisted and thrombocytopenia had developed (45,000/cu.mm) she had her spleen removed. At this time she had had no spontaneous haemorrhage. Again there was no improvement of her haemolytic anaemia but her platelet count returned to normal levels, 310,000/cu.mm.

In January 1957 she was readmitted to Stoke Mandeville Hospital, Aylesbury because of the sudden onset of repeated and severe epistaxes and intractable menorrhagia.

Laboratory Investigations. Hb. 49% (7.25 gms%) Platelets 50,000/cu.mm. Reticulocytes 3.0%. Bleeding time 15+ (Ivy). Direct Coomb's Test on red cells - positive. Both the cold agglutinin and the antibody. Anti-S were still present. Platelet agglutination test was positive against both platelets derived from S+ and S-ve blood to a titre of $\frac{1}{4}$. Prednisolone therapy produced a slow but definite clinical improvement and her platelet count and bleeding time returned to normal limits. Repeated blood

transfusions were also given.

10 days after the start of the Prednisolone therapy a repeat platelet agglutination test was negative.

There had been 3 previous pregnancies, (2 in 1948, 1 in 1954; all resulting in miscarriages at 20, 12 and 14 weeks respectively) prior to the onset of her haemolytic anaemia.

When last seen this patient had no spontaneous bleeding and it was proposed to perform a hysterectomy.

Summary. Thrombocytopenic purpura associated with an acquired haemolytic anaemia in a female aged 54.

52. N.R. (Female) aged 56. In March 1952 this patient was seen, as an out patient, complaining of dryness of the mouth associated with swelling of the parotid gland and vague rheumatic pains in muscles and joints of several months duration. A diagnosis of Sjorgens Syndrome was made and her symptoms were partially improved by cortisone therapy. 2 months later she first noted purpura on her legs and this became more pronounced until, in June 1955, she was admitted to hospital for investigation. Physical examination revealed purpura on the legs, abdomen and one arm. There was marked residual brown staining as the purpura faded. There was no splenomegaly.

Laboratory Investigations. Hb. 82% (12.1 gm%) White cell count 5,000/cu.mm. E.S.R. 23 min/hour. Platelets 89,000/cu.mm. Sternal marrow was normally cellular and normoblastic. Megakaryocytes were easily found. Biochemical examination revealed a cryoglobulin in her serum (0.6 gms%) Platelet agglutination test was negative

at 37°C but positive at 20°C and 4°C with associated precipitation of cryoglobulins. The test was positive when a suspension of the patient's own platelets were used. X-rays and sternal marrow biopsy showed no evidence of myelomatosis.

The purpura continued to appear while in hospital. The platelet count fluctuated between 34,000 and 172,000/cu.mm. Cortisone and 5-hydroxytryptamine creatinine sulphate (Antemovis) failed to improve her purpura.

No previous blood transfusions. One previous pregnancy.

Diagnosis. Thrombocytopenia and purpura associated with cryoglobulinaemia in Sjorgen's Syndrome in a female aged 56.

53. W.P. (Male) aged 49. This man was originally admitted to hospital in 1953 with a 9 months history of a purpuric rash on both legs and lower trunk of 6 months duration. The purpura, when it faded, left marked brown staining of the skin. Physical examination revealed no abnormality apart from this purpura and staining.

Laboratory investigations all gave normal results with the exception of the plasma proteins which showed an excess of Y globulin on electrophoresis. X-ray examination revealed mild splenomegaly. Sternal marrow showed a slight increase in the number of small lymphocytes. No other abnormality was detected. As no satisfactory explanation could be offered for his purpura he was discharged from hospital.

Fresh exacerbation of his purpura necessitated his readmission to hospital in February 1954 when it was discovered that he had an abnormal cryoglobulin in the protein fraction of his blood (1.2 gms%). His E.S.R. was 5 mm/hour at RT. and 45 mm/hour at 37°C. He was never thrombocytopenic but platelet agglutination test at that time was positive (Dr. R. Gillett). At this time the purpura was associated with mild thrombocytopenia (84,000/cu.mm)

In May 1955 he was readmitted because of a cerebrovascular accident which had produced a left hemiplegia. The purpura persisted and a platelet agglutination test performed at this time was negative when performed at 37°C. At 20°C and 4°C marked platelet agglutination appeared to take place along with a dense precipitate of the cryoglobulin fraction. The test was positive when a suspension of the patient's own platelets were used.

This patient's condition subsequently deteriorated and when last heard of he was completely bed ridden due to further cerebro vascular accidents and associated severe hypertension. The purpura was now widespread and involved hands and arms as well as both legs.

No previous blood transfusions.

Summary. Thrombocytopenia and purpura associated with Essential or Idiopathic cryoglobulinaemia in a male aged 49.

54 W.L. (Male) aged 59. In 1947 this man had a severe pneumonia treated by sulphonamides. A few months later he developed purpura of both lower limbs below the knee.

This purpura appeared in successive crops and led eventually to ulceration of the skin. Before these episodes of purpura appeared, the patient felt a burning sensation, and the attacks were exacerbated by cold. If he put his hands or feet into hot water, they became covered with purpura when withdrawn.

Numerous laboratory investigations failed to detect any abnormality either haematologically or biochemically until in 1956 when he was admitted to hospital for reinvestigation.

Physical examination showed extensive purpura of ankles, feet, legs, hands, forearms, and ears. This was associated with marked brown staining of the skin. There was no splenomegaly and no other physical abnormality was detected.

Laboratory Investigations. Hb. 103% (15.2 gms%) W.B.C., 6,000/Cu.mm. Normal distribution. Platelets 234,000/cu.mm. Bleeding time 3'45" (Ivy). C.T. 7'45". Tourniquet test - negative. E.S.R. 37°C - 55 mm/hr, Room temp - 7 mm/hr. 0-4°C 1 mm/hr. Cryoglobulins associated with the Y globulin fraction of the plasma proteins were present at room temperature and in the cold. Marrow biopsy revealed an entirely normal marrow. Platelet agglutination test was negative at 37°C but positive at room temperature. Cortisone therapy exacerbated his purpura and so therapy was stopped. As no other therapy was available he was discharged home.

No previous blood transfusion.

Summary. Purpura associated with Cryoglobulinaemia in a male aged 59.

55. M.W. (Male) aged 56. This man had developed arthralgia of increasing severity over the 3 years prior to admission to hospital in 1954. This was diagnosed as rheumatoid arthritis. Gold therapy was given with no improvement in his condition. He then developed widespread "seborrhoeic dermatitis" which responded to treatment with Phenergan. Subsequently he complained of dysphagia and recurrent sore throats. Immediately before admission he developed bilateral pleurisy with increasing dyspnoea.

Physical examination revealed arthritis in knees, elbows, wrists, shoulders and fingers. There were bilateral pleural effusions and the spleen and liver were enlarged. It was also noted that he had a mask-like facies and the texture of his skin was unduly hard. There was mild ankle and sacral oedema.

Laboratory Investigations. Hb. 87% (12.9 gms%). White cell count 8,500/cu.mm. Platelet count 254,000/cu.mm. E.S.R. 95 mm/hour. Coomb's Test - negative. Urine contained numerous red cells and albumin (1.5 gms/24 hours).

Numerous lupus erythematosus cells were found on every preparation made of peripheral blood. These were also found in smears made from the pleural exudates. Platelet agglutination test was negative. Skin biopsy revealed changes consistent with the diagnosis of scleroderma.

Cortisone therapy improved his arthritis and skin changes and the number of L.E. cells decreased, but were still present 12 months later while clinical remission was maintained by continual cortisone therapy.

No Previous blood transfusion.

Diagnosis. Acute disseminated Lupus Erythematosis in a male aged 56.

56. F.B. (Male) aged 59. This man, who had had severe rheumatoid arthritis for 16 years, developed a concommitant renal lesion with albuminuria and hypertension in 1953. Both his arthritis and renal lesions improved dramatically on cortisone. Physical examination revealed widespread rheumatoid arthritis affecting elbows, knees, wrists, and hands. There was associated ankle and sacral oedema. The spleen was enlarged. There was no evidence of skin lesions and no other significant abnormality was discovered. Repeated examinations for L.E. cells were performed before a positive result was obtained in 1955.

Laboratory investigations revealed a mild anaemia. Hb. 80% (11.8 gms%). White cell count 9-10,000/cu.mm. His E.S.R. was persistently high 90 - 107 mm/hour and has remained high in spite of the clinical improvement obtained with steroid therapy. Total Proteins 5.6 gms%. Albumin 1.6 gms%. Globulin 4.0 gms%. Blood urea, 30 - 52 mgms%. Platelet count 130,000/cu.mm. Coomb's Test - negative. Platelet agglutination test - negative.

No previous blood transfusions.

Diagnosis. Probable acute disseminated lupus erythematosus in a male aged 59.

57. W.P. (Female) aged 54. This woman had developed a progressive lower motor neurone disease following a severe fall 6 years previously. For some years she had noted she

was liable to bruise easily. 3 months before admission to hospital this bruising became more marked and purpura developed but she did not have any other evidence of abnormal bleeding tendency. Apart from large doses of Vitamin E she had taken no drugs in the past 6 months.

Physical examination showed purpura on the anterior chest wall and there were numerous bruises on her arms. There was no splenomegaly. There was wasting of hand and thigh muscles and fibrillation of arm and thigh muscles and the tongue. Upper limb reflexes were brisk; the leg reflexes were depressed ~~but depressed in the legs~~ and the right ankle jerk was absent as were both plantar reflexes. There was no sensory disturbances. There was no evidence of telangiectasia.

Laboratory investigations. Hb. 100% (14.8 gms%) White blood cells 5,600/cu.mm. Platelets 229,000/cu.mm. Bleeding time 15' + (Ivy) was prolonged on the first examination but returned to normal within 2 weeks. Capillary fragility was abnormal. All tests for coagulation function were normal. Coomb's test and platelet agglutination test were negative. It was decided that her bruising and purpura were not connected with her atypical lower motor neurone lesions.

No previous blood transfusion or pregnancy.

Summary. Non-thrombocytopenic purpura in a female aged 54 associated with progressive lower motor neurone disease.

58. R.H. (Male) aged 70. This man was fit until the

age of 52 when he had a sudden melaena. This did not recur until the age of 59 and since then he has had virtually continuous melaena of unexplained origin. A partial gastrectomy was performed 6 years ago and subsequently he has had 3 laparotomies, all of which have not revealed any cause for the persistent gastro-intestinal haemorrhage. He has required multiple transfusions for anaemia secondary to this blood loss. Cortisone and Adrenoxyl therapy produced no improvement. He was admitted to the Radcliffe Infirmary in 1954 when he was found to have a prolonged bleeding time 15' + normal platelet count (405,000/cu.mm.) and abnormal capillaries compatible with Von Willebrand's syndrome. There was no family history of any haemorrhagic diatheses. No other significant abnormality was detected. There was no splenomegaly.

In June 1956 he was readmitted for further investigation as he had had further severe intestinal bleeding requiring blood transfusion. There had been no purpura but there was excessive bruising. Up to this time he had had over 100 blood transfusions.

Laboratory Investigations showed that he was anaemic (Hb. 44% 6.5 gms%) Platelet count 309,000/cu.mm. Bleeding time 9' + (Ivy). Reticulocytes 8.8% Clotting time 5'15". Clot retraction - normal. Patient's platelets generated thromboplastin normally and clot retraction was normal. Capillary fragility was abnormal. Platelet 5 - hydroxytryptamine was abnormally low (5ng/10^8 Platelets). (Normal 60ng/10^8 Platelets). In addition he was found to have an acquired haemolytic anaemia with warm non-specific type antibody (Coomb's test - positive).

Platelet agglutination tests revealed both auto-agglutinins and specific platelet iso-agglutinin, which agglutinated his own platelets and four out of six normal platelet suspensions examined. This antibody was probably the result of his repeated blood transfusions.

As it was felt that this man might be improved by parenteral 5-hydroxytryptamine he was given daily 'Antemovis' (5-hydroxytryptamine - creatinine Sulphate). As there was an apparent improvement in this man's condition, this therapy was continued. While undergoing this treatment this patient was transferred to the Royal Infirmary, Liverpool, and no follow up information has been supplied to date.

('Antemovis' for the treatment of this patient was supplied by Vismara Therapeutici, Como, Italy.).

Summary. Non thrombocytopenic purpura with gastro-intestinal haemorrhage in a male aged 70.

59. J.T. (Female) aged 26. This young woman suddenly developed purpura of both legs below the knee 2 months before she was admitted to hospital in 1953. Shortly afterwards both her ankles started to swell at the end of the day. This latter feature was associated with quite severe pain. Almost simultaneously she developed acute abdominal pain accompanied by diarrhoea but there was no blood seen in the stools. She had had her first child 6 months previously but the pregnancy was entirely normal. There had been no abnormal tendency to bruise.

Physical examination revealed both fading and fresh

purpura lesions of both legs and thighs. There was no splenomegaly.

Laboratory investigations. Hb. 88% (13.0 gms%) Platelets 134-197,000/cu.mm. Bleeding time 3'15" (Ivy). E.S.R. 40 mm./hr. Urine showed mild albuminuria (95 mgms%) with red cells but no casts were present.

Cortisone therapy improved both the purpura and the albuminuria. She was discharged on a maintenance dose of Cortisone for 5 months. Her albuminuria gradually increased and red cells were persistently present in the urine but no casts were seen. Occasional crops of purpura recurred on her legs. When readmitted her platelet agglutination test was negative. The albuminuria gradually became more severe until she was passing 1 gm. protein/100ml urine. The number of red cells present fluctuated from a few to a large number. Her blood urea was never higher than 53 mgm%.

The patient's condition remains the same when last seen in March 1957.

No previous blood transfusion. Previous pregnancy.

Summary. "Henoch Schonlein" non thrombocytopenic purpura in a female aged 26.

60. W.H. (Male) aged 43. This man had been perfectly well until 3 weeks before admission to hospital, when he complained of a sore throat. 10 days later the throat returned to normal but he developed a rash over his back, arms, legs and buttocks. This was accompanied by painful swelling of knees, elbow joints, and hands. To alleviate

the pain, he was in the habit of taking Compound Codeine Tablets.

On admission he was febrile, there was a purpuric rash over his back, arms, legs and buttocks. On examination no other physical abnormality was discovered.

He had persistent albuminuria, with microscopic haematuria. There was no anaemia and the bone marrow was normal in appearance. Megakaryocytes were present. Platelet count 205,000/cu.mm., bleeding time, clotting time, prothrombin time were normal.

Cortisone depressed the E.S.R. to 32 mm/hr. but the purpura and albuminuria persisted until discharge from hospital. Skin biopsy revealed a non-specific vasculitis involving the dermal capillaries and small arterioles.

Platelet agglutination tests were negative. There was no in vitro evidence of drug sensitivity to Aspirin, Phenacetin or Codeine.

No Previous blood transfusion.

Summary. Henoch Schonlein Purpura in a male aged 43.

61. B. (Male) aged 65. This man was referred for haematological investigation following the onset of widespread purpura of legs, arms and trunk of 1 weeks duration. This was associated with recurrent episodes of acute abdominal pain. He had never suffered from any other form of spontaneous bleeding before and had been taking no drugs. He had been travelling extensively abroad and had been flying a great deal.

Physical examination revealed no abnormality apart from purpura.

Laboratory investigation. Hb. 112% (16.6gms%). Platelets 321,000/cu.mm. Bleeding time 3' (Ivy). Capillary fragility - increased. E.S.R. 10 mm/hr. Platelet agglutination test - negative. Urine contained albumin and red cells. No casts were present.

No further history was available in this case as the patient went abroad immediately after examination.

No previous blood transfusion.

Summary. Probable Henoch-Schonlein purpura in a male aged 65.

62. L.B. (Female) aged 77. This woman, a mild diabetic not requiring insulin, was admitted to hospital because of the sudden appearance of widespread purpura associated with abdominal pain and vomiting. Her joints were not involved.

Physical examination revealed a widespread purpura over the feet, legs, thighs, buttocks, lower abdomen, arms, breasts and shoulders. This was confluent in some areas. No other significant abnormality was discovered and there was no splenomegaly.

Laboratory investigations. Hb. 122% (18.0 gms%). White cell count 9,400/cu.mm. Platelets 190-262,000/cu.mm. Bleeding time and capillary fragility were normal. Platelet agglutination test - negative.

The purpura continued to appear in crops affecting the same areas as the initial outbreak.

64. E.W. (Female) aged 24. This young woman, who had had severe ulcerative colitis for one year, was admitted to hospital for the treatment and correction of anaemia resulting from severe blood loss per rectum. She received five separate transfusions of compatible blood and following the last transfusion she developed purpura of the skin over the shoulders, arms, chest and back. She had had no other reaction to the repeated transfusions. She had had one child previously.

Physical examination showed no abnormality apart from the purpura. There was no enlargement of the spleen on the day following the onset of purpura.

Laboratory investigations showed that there was no evidence of thrombocytopenia 239,000/cu.mm. Hb. 64% (9.5 gm%) E.S.R. 96 mm/hr. Bleeding time -4' . Capillary fragility increased. Platelet agglutination tests showed that she had both auto and iso platelet agglutinins to a titre of 1/2. When retested 3 weeks later no demonstrable platelet agglutinins were present.

The case notes of this case could not be found in hospital records and no record was available of any reaction to further blood transfusion.

Summary. Non-thrombocytopenic purpura following blood transfusion in a woman aged 24.

65. M.D. (Male) aged 15. This boy was admitted to hospital with a Type II Nephritis (Ellis) with gross oedema and albuminaemia. There was severe hypo albuminaemia (1-2 gms%) and in an attempt to restore the level to normal

Summary. Allergic Non-thrombocytopenic purpura provoked by human albumen transfusions.

66. J.S. (Female) aged 49. This patient was referred by her general practitioner for investigation of localised purpura of sudden onset. This had appeared 1 week before examination. This purpura had started suddenly and was not itchy or painful. No particular exciting agent could be traced. She had been taking no drugs. Otherwise she had no complaints, and physical examination revealed no abnormality apart from fading purpura over the anterior aspect of both legs.

Laboratory investigations showed that she was not anaemic. Hb. 95% (14.1 gms%). White cell count 7,800/cu.mm. Platelets 217,000/cu.mm. Bleeding time 9' (Ivy). Prothrombin time (Quick) Normal 15". Patient 15". Peripheral blood - Normal. Capillary fragility - increased. Platelet agglutination test - Negative.

No further history or follow up was available.

No previous blood transfusions or pregnancy.

Summary. Non-thrombocytopenic purpura in a female aged 49.

67. P.C. (Female) aged 17. This girl had a 'port-wine' naevus of her left leg below the knee which was a true vascular 'hamartoma'. Plastic surgery was attempted for cosmetic reasons but, the tumour having been removed, no healing took place and a series of skin grafting operations failed. She was investigated haematologically because of

and thus control the oedema, 4 transfusions of human albumen (75 gms/transfusion) were given. One month later they were repeated but this time there was a marked reaction to each transfusion with vomiting, rigors and unexplained bouts of coughing. After one of these reactions, scattered purpuric haemorrhages were noted and at the same time his red cells gave a positive direct Coomb's Test. There was no thrombocytopenia (Platelets 207,000/cu.mm). The bleeding time and capillary fragility were normal.

Investigations by in vitro platelet agglutination tests showed that blood taken immediately after a transfusion of human albumen contained an auto-agglutinin against the patient's own platelets. The blood taken 2 days later showed no such auto-agglutinin but, on the addition of human albumen (Lister Inst.) to platelet rich plasma, platelet agglutination occurred and clot retraction was inhibited. Similarly the patient's serum, which did not agglutinate normal platelets by itself, did so very strongly when human albumen (Lister Inst.) was added. Preparations of the patient's own albumen, American Human Albumen, Bovine, used in similar concentrations (Experiment 44) did not provoke agglutination. (Appendix B, p.35).

In view of these reactions to albumen no further transfusions were given. 1 month later platelet agglutination test were entirely negative.

Prednisolone therapy produced a clinical remission with a total disappearance of the albuminurea and oedema. This has been maintained for 1 year.

One previous blood transfusion.

this failure of skin grafts to take in the wound edges, an unexplained ooze of blood from the wound and some purpura of the skin surrounding the wound.

Physical examination showed that the spleen was not enlarged.

Laboratory investigation revealed that she was mildly anaemic. Hb. 76% (11.2 gms%) White cell count and differential were normal. Platelet count was normal 206,000/cu.mm. Bleeding time and capillary fragility were within normal limits. There was no evidence of any haemolytic process and no abnormal proteins were detected. Her erythrocyte sedimentation rate was raised 79 mm/hr. Capillary microscopy showed marked clumping of the red cells in the circulation. This is an abnormal finding but no explanation could be offered for this anomally. No diffuse or focal telangiectasis could be detected. Platelet agglutination test was negative. Cold agglutinin titre 1/16.

The original wound began to heal very slowly but 9/12 later it had not yet completely healed and within the clear tissue that had formed, obvious telangiectasis of small capillaries were present.

One previous blood transfusion.

Diagnosis. Non-thrombocytopenic purpura associated with an unexplained failure to heal following excision of vascular hamartoma of leg.

68. S.H. (Female) aged 5. This child was investigated because of unexplained intraocular haemorrhage involving the anterior chamber of the eye. No abnormality was discovered

apart from abnormal capillary fragility. Hb. 95% (14.1 gms%) Platelets 216,000/cu.mm. Platelet agglutination tests - negative. B.T. (Ivy) 4'.

No previous blood transfusion.

Summary. Unexplained intraocular haemorrhage in a female aged 5.

69. M.G. (Female) aged 38. This woman had bruised easily for several years and had latterly suffered from severe menorrhagia. In October 1953 she developed a purpuric rash appearing first on the dorsum of the foot and spreading over the trunk and arms. This persisted for 1 year and in January 1954 a cervical biopsy produced a marked secondary haemorrhage. The purpura persisted and, as the patient herself felt that it was exacerbated by phenobarbitone, she was admitted to hospital for investigation. Physical examination revealed purpuric haemorrhages mainly over the feet, ankles and buttocks but there were a few on the trunk and thighs. There was no splenomegaly.

Laboratory investigations revealed a normal platelet count (312,000/cu.mm) and normal bleeding time and capillary fragility. All tests of coagulation function were normal and platelet agglutination tests were negative. Tests for drug sensitivity to phenobarbitone revealed no positive results. (Appendix A, p.16).

Capillary Microscopy (Dr. R.G. Macfarlane) suggested that the purpura was unusual in that the area of extravasated blood appeared to be surrounded by a zone of congested capillaries. "Adrenoxyl" (Adrenochrome mono semicarbazome) was given but produced no improvement of

the purpura.

No previous blood transfusion. Previous pregnancies.

Summary. Non-thrombocytopenic purpura in a woman aged 38.

70. S.B. (Female) aged 51. For 2 months prior to examination this patient had developed an acute irritant rash of her forearms, waist and groins. This had a strong purpuric element to it and the skin was hyperkeratotic.

Physical examination showed numerous scratch marks over the affected parts. No other physical abnormality was detected.

Laboratory investigations. Hb. 113% (16.7 gms%). White blood cells 5,100/cu.mm. Total neutrophils 3,000/cu.mm. Bleeding Time 3' 15" (Ivy). Capillary Fragility: markedly increased. Platelets: 189,000/cu.mm. Platelet agglutination test - negative.

No previous blood transfusions or pregnancy.

Summary. Non-thrombocytopenic purpura in a female aged 51.

71. A.F. (Female) aged 46. This woman suddenly developed purpura over her legs and arms, which continued to appear sporadically over the next 2 months. She had been taking no drugs.

Physical examination revealed no abnormality apart from the purpura.

Laboratory investigations. Hb. 100% (14.8 gms%)

B-globulin fraction.

3 weeks later the platelet agglutination test was negative but the abnormal protein pattern persisted.

No previous blood transfusion.

Summary. Positive platelet agglutination test in a case of Virus Pneumonia.

APPENDIX D

STATISTICAL ANALYSES

(I am indebted to Mr. N.T.J. Bailey of the Department of Biometry, University of Oxford for his guidance in the preparation of this Appendix)

In Part II of this thesis the appraisal of the results of platelet agglutination tests on normal and abnormal bloods necessitated statistical analyses in order to establish whether these results supplied information of value or were due to chance observation.

In all examples this involved the straight-forward analyses of four groups of related results and thus it was convenient to use the following type of χ^2 (Chi-squared) test for this purpose.

The experimental results were tabulated according to the following table:

a	b	a + b
c	d	c + d
a+c	b+d	n = Total

Where a, b, c and d represent the number of observations in each group, and n the total number of observations.

With the information derived from this table X^2 was determined by the following formula, using Yates' correction,

$$\chi^2_{(1)} = \frac{n\{|ad-bc| - \frac{1}{2}n\}^2}{(a+b)(c+d)(a+c)(b+d)}$$

(Bradford Hill, 1942).

$\chi^2_{(1)}$ having been determined, the value P was determined for one

degree of freedom (n = 1) using the X^2 tables (R.A. Fisher, 1936. Statistical Methods for Research Workers, 6th Edition. Oliver and Boyd, Edinburgh and London).

It is satisfactory in certain biological experiments to accept results as significant when the associated value of P is less than 5%, and the value of X^2 corresponding to a significance level of 5% (P, 0.05) is 3.841 for one degree of freedom.

Analyses of Experimental Results.

A. Analyses of results obtained from Table XL

The numbers of positive platelet agglutination tests were sub-divided with groups according to their ABO compatibility or incompatibility.

	Positive Tests	Negative Tests	Total Tested
ABO Compatible Platelet-serum Mixtures	29	668	697
ABO Incompatible Platelet-serum Mixtures	43	260	303
	72	928	1000

$X^2_{(1)} = 30.3$

Therefore for one degree of freedom $P < 0.01$

B. Analyses of results obtained from Table XXXIX

(i) The numbers of positive platelet agglutination tests in the following groups were analysed.

(a) Platelets derived from Group O blood cross matched with serum derived from Group O, A and B blood.

(b) Platelets derived from Group A and B blood cross matched with serum derived from Group A and B blood respectively

	Positive Tests	Negative Tests	Numbers Tested
Group O Platelets + Group O, A, B serum	12	458	470
Group A or B Platelets + Group A or B serum	17	210	227
	29	668	697

$X^2_{(1)} = 8.15$

Therefore $P < 0.01$

(ii) The number of positive agglutination tests were analysed in the following groups.

(a) Platelets derived from blood Rhesus group positive and negative were cross matched with sera compatible in terms of ABO groups.

	Positive Tests	Negative Tests	Numbers Tested
Group Rh +	20	292	312
Group Rh -	2	135	137
	22	427	449

$X^2_{(1)} = 4.0$

$P < 0.05$

C. An analysis of the positive agglutination tests in thrombocytopenia and non-thrombocytopenic syndromes

The differences between the above two groups were compared using data derived from Table XLIX .

	Positive Tests	Negative Tests	Total Tests
Thrombocytopenic group	23	32	55
Non-Thrombocytopenic group	6	12	18
	29	44	73

$\chi^2_{(1)} = 0.13$

$P > 0.70$

D. An analysis of positive agglutination tests in relation to splenic enlargement. (Data obtained from Table L)

The pattern of results, in relation to the size of the spleen, could be expressed as follows:

	Positive Tests	Negative Tests	Total Tests
Normal spleens	15	36	51
Enlarged spleens	9	11	20
	24	47	71

$\chi^2_{(1)} = 0.94$

$P > 0.30$

E. Analyses of results obtained from Tables LI and LII relating positive platelet agglutination tests to the response to Steroid therapy and Splenectomy

The pattern of results could be expressed as follows:

(i) The relationship of positive platelet agglutination tests to the response to Steroid therapy

	Positive Tests	Negative Tests	Numbers Tested
Response	4	8	12
No response	3	7	10
	7	15	22

(ii) The relationship of positive platelet agglutination tests to the response to Splenectomy

	Positive Tests	Negative Tests	Numbers Tested
Response	4	3	7
No response	2	3	5
	6	6	12

In each of these tables there is at least one entry having an expected value of less than five observations, e.g. the lower left expectation in the table relating to splenectomy is 6x5 / 12 = 2.5. Therefore, it was not possible to apply the X^2 test to these results. While statistical analysis by other methods could have been done, it was apparent from the inspection of the tables that the differences between the groups were not likely to achieve significance. Therefore, it can be concluded there was no correlation between the positive platelet agglutination tests, and the response to Steroid therapy or Splenectomy.

F. Analysis of results obtained from Tables LIII A & B

This information was analysed in the two sub-divisions detailed in this table.

(i) The relation of positive platelet agglutination tests to blood transfusion.

(ii) The relationship of positive platelet agglutination tests to pregnancy.

Blood Transfusion

	Positive Tests	Negative Tests	Numbers Tested
Previous blood transfusion	10 (a)	13 (b)	23
Never received blood transfusion	19 (c)	29 (d)	48
	29	42	71 (n)

$\chi^2_{(1)} = 0.003$

$P > 0.95$

Pregnancy

	Positive Tests	Negative Tests	Numbers Tested
Previous pregnancy	15 (a)	14 (b)	29
Never pregnant	14 (c)	30 (d)	44
	29	44	73 (n)

$\chi^2_{(1)} = 2.12$

$P > 0.10$